EXPRESSION OF LEPTIN IN HUMAN DENTAL PULP AND ITS ROLE IN ACUTE AND CHRONIC PULPAL PATHOSIS

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DECLARATION BY THE CANDIDATE

I hereby declare that this dissertation titled **"EXPRESSION OF LEPTIN IN HUMAN DENTAL PULP AND ITS ROLE IN ACUTE AND CHRONIC PULPAL PATHOSIS "** is a bonafide and genuine research work done by me under the guidance of **Dr. R. ANIL KUMAR M.D.S.,** Professor & Head of the Department of Conservative Dentistry and Endodontics, Ragas Dental College and Hospital, Chennai.

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LIST OF ABBREVATIONS

S. NO.	ABBREVATIONS	DESCRIPTIONS
1	α	Alpha
2	β	Beta
3	IL	Interleukin
4	TNF	Tumor necrosis factor
5	qT-PCR	Quantitative Real-time Polymerase Chain Reaction
6	RNA	Ribonucleic Acid
7	DNA	Deoxyribonucleic Acid
8	mg	Milligram
9	kg	Kilogram
10	CD	Cluster of Differentiation
11	ELISA	Enzyme-linked immunosorbent assay
12	BMI	Body Mass Index
13	LPS	Lipopolysaccharide
14	ob	Obesity gene - leptin
15	Ob-R, LEPR	Leptin receptor
16	db	Diabetes gene

17	DSPP	Dentin Sialophosphoprotein
18	rpm	Rotations per minute
19	°C	Degree Celsius
20	cDNA	Complementary DNA
21	CT or CP	Crossing threshold / Crossing point
22	ANOVA	Analysis of Variance
23	SPSS	Statistical Package for Social Sciences software
24	DC	Dendritic cells
25	NK	Natural killer cells
26	IFN	Interferon
27	DPSC	Dental pulp stem cells
28	Ig	Immunoglobulin
29	>	Greater than
30	<	Less than
31	%	Percentage
32	#	Size

Introduction

INTRODUCTION

Connective tissue is a fibrous type of supporting tissue with numerous functions widely distributed throughout the body. The major function of connective tissue is to provide a matrix that binds the cells and organs, ultimately giving support to the body. It is also responsible for various activities that initiate and orchestrate reactions to pathogenic invasion, and thus serves as an essential site for host defense.[127]

The dental pulp is a richly innervated, highly vascularized, soft and loose connective tissue of mesenchymal origin that is residing within the pulp chamber and root canal of the tooth and is uniquely surrounded by the rigid encasement of mineralized dentin. However there are certain unique features for the dental pulp that distinguishes it from the other connective tissues. Under normal conditions the connective tissue dental pulp is sterile and isolated from the oral microbiota by overlying enamel, dentin and cementum which act as a rigid physical barrier against the injuries of external environment and pathogenic challenges[54]. This peculiar anatomic location of the dental pulp and the scarcity of collateral circulation render it more susceptible to injury and complicate its healing, repair and regeneration.[86]

When the integrity of this rigid hard tissue barrier is breached and exposed to oral environment, it is challenged by the noxious elements of external origin. The progression of bacteria from the outermost enamel to the

pulp–dentin interface triggers inflammatory and immune events in the underlying dental pulp through the diffusion of bacterial by-products into the dentinal tubules. The inflammation of dental pulp is termed as Pulpitis. There are various factors that induce inflammation in pulp. In majority of cases, it is due to the ingression of microorganisms into the pulp space as a result of dental caries, traumatic fractures or injuries, dentinal cracks, exposed dentinal tubules, mechanical irritation during tooth preparation or cavity preparation, trauma from occlusion, chemical irritation from etching materials, and orthodontic movement of teeth.[86]

Inflammation is the consequence of complex interactions among various cell types. These events may be prevented when reactionary or reparative dentin is formed by odontoblasts at the pulp-dentin interface that eliminates the bacteria and blocks the route of infection[28]. In the absence of odontoblast reaction or in case of odontoblast death, bacterial invasion leads to irreversible pulpitis, pulp necrosis, infection of the root canal system, and periapical disease (Love and Jenkinson, 2002).[74]

The disease progression in the dental pulp follows the following sequence – normal pulp, inflammation (pulpitis), necrosis, infection, and loss of pulp tissue inside the root canal. The degree of inflammation is comparable to the severity and the duration of stimulus, as well as the host's capacity to defend the pulpal response.[46]

Pulpitis can be reversible or irreversible based on the healing potential. A "clinically normal pulp" is a pulp that does not present with any signs and symptoms (Abbott PV 2007)[2]. Pulpal pain is usually the first clinical sign of pathology. Reversible pulpitis is characterized by mild to moderate inflammation in which the pulp is capable of returning to uninflammed state following the removal of stimuli. If the noxious stimuli is not removed the inflammation progresses to persistent irreversible pulpitis. This is a persistent inflammatory condition of pulp which can be acute or chronic based on the subjective and objective findings.

It is well established that the normal dental pulp contains heterogeneous cell populations including a majority of fibroblast-like cells, latent or dormant pulp stem cells (progenitors) which are mostly involved in self-renewal. In addition pulp is armored with several biological factors like defense immune and inflammatory cells and is capable of expressing a large number of known host mediators of inflammation like cytokines and chemokines, as it is evidenced by their identification in pulp at the gene-expression levels.[95] The principal objective of these mediators is to combat the irritating factors, to mount immune responses against the invading cariogenic bacteria and minimize their harmful effects.

Cytokines are large group of small secreted soluble proteins that act as extracellular signaling molecules. They act as messengers between immunocompetent cells and, act as mediators and regulators of inflammatory

responses. They are actively involved in the reparative and destructive events in the pulp by its elevated expression and stimulation of cellular and molecular agents participating in pulpal inflammatory cascades. Hence their overall balance must be maintained to modulate balance between humoral and cell based immunity. Some of them are namely chemokines, interleukins IL, lymphokines, adipokines, etc.,[46]

Immunity is the capability of multicellular organisms to resist harmful microorganisms from entering it. The bacterial elements potentially activate various forms of immune reactions by acting as antigens. Upon initial invasion of microbes, non-antigen specific innate immunity is activated to rapidly eliminate the bacterial irritants followed by antigen specific adaptive immunity to enhance the protective mechanism. The inflammatory reaction is characterized by the presence of various immune cells namely neutrophils, monocytes, macrophages, lymphocytes, dendritic cells which can produce inflammatory mediators such as pro-inflammatory cytokines like IL-1β,IL-2,IL-6,IL-8,TNF-α.[42]

It is also recognized that there is no clear-cut difference between inflammation and immunity. Acute inflammation, innate immunity, humoral immunity and cell-mediated immunity together provide the host with a wide array of weapons with which to combat pathogens and bring about healing. The major role of these systems is to protect the individual from invasion by infectious organisms that can cause disease.

Adipose tissue was previously believed to be only a lipid storage organ. In recent days, adipose tissue gained more attention after demonstration of secretion of various bioactive substances called adipokines by adipocytes. These adipokines are family of cytokines that includes leptin, IL-6, adiponectin, resistin, TNF-α. The balanced secretion of these bioactive substances is essential for the maintenance of energy homeostasis.[26]

Leptin, 16KDa non-glycosylated polypeptide hormone of 146 amino acids predominantly produced by adipocytes was discovered in 1994. The term Leptin originated from a Greek word 'Leptos' meaning 'thin'. This adipokine encoded by ob gene is a product of obesity gene produced in adipose tissue in a central manner via its cognate receptor in hypothalamus. Various studies have reported the role of leptin in regulation of energy homeostasis and metabolism i.e., intake and expenditure, appetite and hunger in a central manner.[104] It is also produced in minor quantities by placenta, stomach, lymphoid tissues, salivary glands. Apart from energy homeostasis leptin has systemic effects, including regulation of neuroendocrine, reproductive, haematopoietic and immune function.

Leptin is considered as a pleiotrophic molecule that plays a dual role as a hormone and a cytokine. Besides playing an important role as a hypothalamic modulator of food intake, fat storage and regulation of body weight, recent studies showed that leptin also acts as a proinflammatory cytokine. Various studies have reported that leptin and its receptor share

structural and functional similarities with members of long chain helical cytokines like IL-1,IL-6,IL-10,IL-12,granulocyte stimulating factor and activates few proinflammatory signaling pathways, resulting in the induction of several biological markers of inflammation.

Researches in the past few years have highlighted the intricate network that links immune homeostasis, nutrition and neuroendocrine systems. Studies have evidenced the presence of leptin in inflamed and healthy human dental pulp tissue samples and the results showed that leptin is up-regulated in inflamed pulp tissue compared to healthy tissue suggesting its role in pulpal inflammatory and immune responses.[72]

There is an apparent lack of clear understanding in pulpal inflammation and its relation to pulpal immune responses. The numerous genome sequencing projects conducted in the last few years have furnished information on the importance of immune systems during inflammation. With the advancements in microarray technologies, have provided various diagnostic tools to understand the biology of these molecular events working during inflammation. Hence the quantitative real time PCR (qt-PCR) analysis of the production of the inflammatory cytokine, leptin in various inflammatory conditions of pulpitis helps to expound the immunopathologic mechanism of pulp. The knowledge acquired can then be applied to immunotherapy of inflamed pulp in endodontic treatment.

Aim and Objectives

AIM AND OBJECTIVES

AIM:

To describe and analyse the possible expression of Leptin in human dental pulp, and its role in acute and chronic pulpal pathosis.

OBJECTIVES:

1. To evaluate the expression of leptin in the healthy and inflamed human dental pulp samples by quantitative real-time PCR assay.
2. To compare and analyze the variations in the relative expression of leptin in healthy, acute and chronic pulpal pathosis groups

Review of Literature

REVIEW OF LITERATURE

Zhang et al (1994)[118] discovered the mouse *ob* gene through positional cloning and was shown to encode a 4.5 kilobase mRNA transcript with a highly conserved 167 aminoacid open reading frame that was unique in the GenBank database. They stated that one of the molecules that regulates energy balance in the mouse is the obese (*ob*) gene. The ob gene product that may function as a part of a signaling pathway from adipose tissue that acts to regulate the size of the body fat depot

Considine et al (1996)[17] correlated serum leptin levels in obese and normal weight individuals using radio immune assay. One hundred and thirty nine normal-weight subjects and one hundred and thirty nine obese subjects were categorised according to their mean body mass index. The results showed that there was a positive correlation between serum leptin and body fat mass. Higher concentration of leptin was found in obese women than in obese men and increased plasma leptin level with increase in body mass index. Females showed increased serum leptin concentration with increase in body mass index when compared to males of same age. This correlation suggested that adipocytes are signaling the brain about the size of the adipose-tissue depot.

Sinha KM et al (1996)[102] investigated the effects of free and bound leptin in human circulation. Forty six lean and obese subjects were selected and serum samples were collected and fractioned using gel filtration technique. Leptin levels were detected using radio immunoassay. The results of the study showed that total leptin levels were increased significantly in

obese subjects when compared with lean individuals. Majority of leptin circulated as a bound form in lean individuals, on the contrary in obese subjects the majority of leptin circulated as free form. This study further investigated the effects of fasting on free and bound leptin ,where they found out that fasting had a significant effect in free form in obese individuals than bound form in lean individuals

Masuzaki H et al (1997)[73] demonstrated the production of leptin by placenta. Blood samples were obtained from pregnant and non-pregnant women before and after delivery. Amniotic fluids were also collected at the time of caesarean section at various gestational stages. Primary culture of human amnion cells were prepared from amnion tissue. Human leptin levels in plasma, amniotic fluid and cultured medium from amnion cells were determined by radioimmunoassay kit. The results showed that plasma leptin levels were markedly elevated in pregnant women than in non- pregnant women. Leptin were also produced by primary cultured human amnion cells. These evidence suggested that placenta acts as a substantial source of circulating leptin during pregnancy and hence it could be a placenta derived hormone.

Tasaka Y et al (1997)[108] evaluated human plasma leptin in obese and diabetic individuals using radio immunoassay. The results revealed cholecystokinin-8 and killed after fifteen minutes. Leptin content was analysed. Exogeneous administration of cholecystokinin resulted in decrease in leptin content of fundic epithelium with subsequent increase in

concentration of leptin in plasma. These results indicated that gastric leptin might involve early cholecystokinin mediated effects activated by food intake.

Bado et al (1998)[7] explored the presence of leptin protein in fundic epithelium of stomach. Fundic epithelial scrapings were obtained from wistar rats and the epithelium were homogenised and centrifuged. The resultant supernatants were used for leptin analysis. In another set of experiments, fasted rats were injected intraperitoneally withnormal weight men. But there was no significant difference between the gender, when comparing women and men with equivalent percentage of body fat.

Faggioni et al (1998)[24] conducted an animal study to investigate the role of leptin in induction of cytokines during inflammation. Mice of mixed variety were injected intraperitoneally with lipopolysaccharide at a dose of 5mg/kg and with turpentine subcutaneously in the hind limb. Control mice were injected subcutaneously or intraperitoneally with sterile pyogen free saline. Blood samples and epididymal tissues were removed six hours and sixteen hours after treatment and serum leptin levels were measured using enzyme linked immunosorbent assay. The results showed that both lipopolysaccharide and turpentine increased leptin mRNA and circulating leptin in interleukin 1 beta mice. But in interleukin deficient mice either lipopolysaccharide or turpentine increased leptin level. Turpentine increased leptin in interleukin -6. This study concluded that interleukin 1 beta has a role in induction of leptin during inflammation.

Loffreda (1998)[65] conducted experiments on rodents with genetic abnormalities in leptin and leptin receptors. He revealed the rodents had

obesity related deficits in macrophage phagocytosis and expression of proinflammatory cytokines both in-vivo and in-vitro. Exogenous leptin administration upregulated both phagocytosis and production of proinflammatory cytokinesthese results identified leptin upregulates proinflammatory immune responses

Fantuzzi G et al (2000)[27] in their review stated that leptin is a product of obese gene that regulates food intake as a metabolic hormone. It also plays a major role in immunity, inflammation and hemotopoiesis. Both leptin and its receptor share structural and functional similarities with members of long chain helical cytokines like IL-1,IL-6,IL-12, granulocyte stimulating factor. Author further suggested that leptin modulates cytokine production from monocytes and its production is increased during inflammation and infection.

Steppen et al (2000)[103] investigated the role of exogeneous leptin in stimulation of bone growth when administered in leptin deficient mice using x-ray absorptiometry. The outcome was that total mineral content both trabecular and cortical bone was increased. Leptin receptor was expressed in both primary osteoblasts and chondrocytes suggesting a direct effect of leptin on bone growth as a signaling molecule in bone remodelling.

Alhashimi N et al (2001)[4] explored the effects of orthodontic force to induce denovo synthesis of pro-inflammatory cytokines like interleukin-6, interleukin-1 β which played a important role in bone resorption, bone remodeling and new bone deposition. In this study in situ hybridisation technique with subsequent counting of mRNA expression was used for

detection of cytokines. The results of the study showed that proinflammatory cytokines were released after application of orthodontic force.

Johnson RB et al (2001)[52] studied the levels of leptin, vascular endothelial growth factor, interleukin -6 in healthy and diseased human gingival tissues. Tissue samples were collected from healthy and inflamed gingiva. Their concentration was assessed with solubilised gingival biopsies by enzyme linked immunosorbent assay.Results showed that leptin concentrations in gingiva adjacent to 4 -6 mm pocket depth as compared to gingival tissues adjacent to less than 3mm sulcus. Vascular endothelial growth factor and interleukin-6 concentration negatively correlated with leptin concentration. Authors concluded that leptin was present in both healthy and inflamed gingiva and release of leptin from gingiva could be coincident to vascular expansion. Gingiva in addition to adipose tissue could be a source of circulating leptin.

Wallace et al (2001)[112] investigated plasma leptin as a risk factor for cardiovascular disease. This case control study measured plasma leptin levels in subjects (cases) who experienced a coronary event and in subjects(controls) who remained free of an event during the five year follow up period of the study. Concentration of leptin was found to increase in cases than control group and it correlated with C- reactive protein levels indicating that leptin could be a risk factor for coronary heart disease. The authors further concluded that leptin has a role in immune function in association with C-reactive protein.

Sánchez-Margalet et al (2003)[96] investigated the role of leptin in blood mononuclear cells. Leptin receptor is expressed in human peripheral blood mononuclear cells, mediating the leptin effect on cellular proliferation and activation. In vitro activation and HIV infection in vivo induce the expression of the long isoform of the leptin receptor in mononuclear cells. Also, leptin stimulates the cultured monocytes and enhances the production of proinflammatory cytokines and Th1 type cytokines from stimulated lymphocytes.

Shalitin S et al (2003)[100] in their review investigated the role of obesity and leptin in pubertal process and pubertal growth. Obese children have high leptin levels and this degree of body fatness may trigger the neuroendocrine events that lead to the onset of puberty. Overweight children especially girls tend to mature earlier than lean children. Children with obesity also have increased adrenal androgen levels, which may involve in the accelerated growth of children before puberty. Authors further concluded that obesity is associated with early puberty and elevated leptin levels might have a permissive effect on the pubertal process and pubertal growth.

Antonio La Cava et al (2004)[60] compared leptin as a neuroendocrine and immune mediator and indicated that, the mediators common to the neuroendocrine and immune systems, such as the cytokines interleukin-1 (IL-1), IL-6, tumour necrosis factor (TNF) and leptin can all modulate inflammation through the HPA(hypothalamo-pituitary -adrenal) axis. Leptin along with C reactive protein acts as an early acute-phase reactant, which are produced at high levels during inflammation

Giuseppe Matarese et al (2005)[74] correlated the role of leptin in the pathophysiology of immune responses. In humans, leptin regulates energy homeostasis and neuroendocrine function primarily in states of energy deficiency. Leptin, a cytokine in addition modulates thymic homeostasis , similar to other proinflammatory Cytokines.

Meyers AJ et al (2005)[79] in their review presented a summary relating role of serum leptin concentration in immunity and inflammation. In vitro and animal studies suggested a complex role of leptin in immune function, including associations with natural killer cells (NK cells), C-reactive protein, tumor necrosis factor alpha, interleukin -6, serum amyloid A. Few studies suggested a direct link between cytokines and leptin.

Bozkurt et al (2006)[11] evaluated the effects of smoking on gingival crevicular leptin levels in periodontitis patients. One hundred and forty three subjects were selected and divided into three groups; non smokers, smokers and control group. Probing depth and clinical attachment loss values were collected from maxillary anterior teeth using Williams probe. Gingival crevicular fluid samples were collected from maxillary anterior teeth using paper strips. Gingival crevicular fluid leptin levels were determined using enzyme linked immune sorbent assay. They observed that gingival crevicular fluid leptin levels were decreased in smokers group when compared to non smokers group. This study concluded that higher leptin levels in gingival crevicular fluid might play a protective role in periodontitis patient.

Karthikeyan B and Pradeep AR (2007)[55] explored leptin levels in gingival crevicular fluid in periodontal health and disease. Subjects were

divided into two groups; one group with healthy gingiva and the other group with periodontal disease. Gingival crevicular fluid samples were col lected from both groups using micropipettes. Leptin concentration in gingival crevicular fluid were analysed using highly sensitive enzyme linked immune sorbent assay kit. The results showed that leptin concentration in gingival crevicular fluid was decreased as periodontal disease progressed. They also reported that there was a strong negative correlation found between gingival crevicular fluid leptin levels and clinical attachment loss.

Lago et al (2008)[62] in their review stated that leptin is a 16KDa polypeptide hormone produced primarily by adipocytes. Leptin plays a dual role as a hormone and cytokine. As a hormone it plays a major role as a hypothalamic modulator of food intake, fat storage, body weight and as a cytokine it plays a major role in immune and inflammatory response. Author further probed that leptin also play a role in the pathogenesis of autoimmune inflammatory conditions such as encephalomyelitis, type I diabetes, bowel inflammation and also articular degenerative diseases such as rheumatoid arthritis and osteoarthritis.

Groschl et al (2009)[36] studied in healthy individuals to identify the presence of leptin in saliva and its stability under gastric conditions. They collected plasma and saliva from sixty subjects and leptin in salivary glands and oral mucosa were identified by real time polymerase chain reaction. The results showed that leptin will be present in saliva and there was a strong linear correlation between plasma and salivary leptin. Specific leptin receptors

are located in the gastric mucosa of rats and humans and in intestinal cells of mice

Martin-Gonzalez et al (2009)[72] investigated leptin expression in healthy and inflammed human dental pulp from pulp samples of third molar using quantitative real time polymerase chain reaction and amount of leptin was calculated using immunoblot assay. The results of the study showed that leptin can be expressed in healthy and inflammed human dental pulp and leptin expression was twice more in inflammed pulp when compared to normal pulp samples.

Stofkova A (2009)[104] in their review discussed the contribution of leptin and adiponectin in inflammatory and autoimmune processes with particular interest in rheumatoid arthritis. Adipose tissue plays a critical role as an endocrine organ in the regulation of immune and inflammatory processes, which produces number of active peptides called adipokines. They highlighted the importance of leptin in the pathogenesis of rheumatoid arthritis.

Bruno (2010)[13] evaluated the microscopic characteristics of inflammatory cell infiltrate in human dental pulpitis. Human dental pulp collected from 38 human teeth with irreversible pulpitis were removed by pulpectomy and healthy pulps from orthodontically extracted teeth which were used as controls. The pulp tissue was immersed in 10% buffered formalin for evaluation using light microscopy. The control group showed significantly lower numbers of CD68+ macrophages, CD20+ B lymphocytes, CD4+ T helper lymphocytes, and CD45RO+ memory T lymphocytes when compared to irreversible pulpitis group.

Haghighi. AK (2010)[40] in his study evaluated the presence and concentration of Leptin in chronic periapical lesions. The study includes chronic periapical lesions with different sizes which were collected during periapical surgery of the mandibular molars from twenty patients and cultured for 72 hours. The ELISA method was used to determine the concentration of Leptin in supernatant fluids of explants culture. Results indicated that Leptin was found in all samples with the average concentration of 405.55±102.98 (pg/mL). He added that there was no significant correlation between the concentration of Leptin and BMI, and the diameters of lesions and concluded that leptin have a role during the early phases of dental periapical lesions.

Patricia Fernandez-Riejos et al (2010)[30] stated leptin is a mediator of the inflammatory response and correlates the role of leptin in the mechanisms of immune response and host defense. They concluded that the circulating leptin levels are increased upon infectious and inflammatory stimuli such as LPS, turpentine, and cytokines

Agrawal et al (2011)[3] investigated the role of leptin in activating human B cells to secrete proinflammatory cytokines like interleukin-6, interleukin - 10 and tumor necrosis factor alpha. Purified B cells were incubated with various concentration of leptin for twenty four hours and cytokines were measured in the supernatants by enzyme linked immunosorbent assay. The results indicated that leptin stimulates secretion of cytokines via phosphorylation of Janus Activation Kinase 2(JAK2),signal transducer and activator of transcription 3(STAT 3), p38 mitogen- activated protein kinase (p38 MAPK) and extracellular signal regulated kinase (ERK)

pathway which might contribute to its role in inflammation and immuno-regulatory response.

Paul FR et al (2011)[88] evaluated serum leptin concentration with body mass index. Two hundred and seventy samples were categorized into three groups based on their body mass index as normal, overweight and obese. Blood samples were collected and serum leptin levels were analyzed. Obese patients had seven times greater serum leptin levels and overweight patients had four times greater serum leptin levels than normal weight patients. When compared to men women had increased serum leptin levels. However there were no differences between men and women if equal percentage of body fat were taken into consideration.

Ay.ZY (2012)[6] investigated the presence of leptin and its receptor in serum and in the gingiva of healthy controls, gingivitis, aggressive periodontitis and chronic periodontitis patients. He correlated the clinical and serum parameters by collecting blood sample, gingival tissues obtained. Serum parameter levels were assessed with ELISA and OB (leptin) and OB-R (leptin receptor) in gingiva by immunohistochemically. In all of the tissue samples of all groups, there was positive OB and OB-R. The serum levels of OB and s-OB-R donot vary between patients with different periodontal conditions

Martin Gonzalez (2013)[70] analyzed the expression of leptin in human periapical granulomas. Fifteen periapical inflammatory lesions were obtained from teeth which underwent periapical surgery and extracted human teeth . The periapical lesions were examined by immunohistochemistry using human

leptin polyclonal antibodies. Quantitative real-time PCR (qRT-PCR) was used to determine the expression of Leptin mRNA. He concluded that Quantitative RT-PCR analysis confirmed the expression of leptin mRNA and the size of the amplified fragment (296 bp for leptin and 194 bp for cyclophilin) was assessed by agarose gel electrophoresis. Western blot analysis revealed the presence of protein with apparent molecular weight of approximately 16 kDa, corresponding to the estimated molecular weights of leptinin all the samples

Martin Gonzalez (2015)[71] evaluated the immmunohistochemical localization of LEPR (leptin receptor) and the effect of leptin on DSPP (dentin sialophosphoprotein) expression in human dental pulp. Dentin sialophosphoprotein (DSPP) is involved in dentinogenesis and the dental pulp reparative response. Twenty-five dental pulp specimens were obtained from freshly extracted human third molars. Immunoreactivity for LEPR concentrated in the odontoblast layer. Leptin dose dependently stimulated DSPP expression. The expression of DSPP messenger RNA was confirmed by quantitative realtime polymerase chain reaction, and the size of the amplified fragments (298 bp) was confirmed by agarose gel electrophoresis. These findings further support the functional role of leptin in the dentin mineralization process and/or in dental pulp reparative and immune responses.

Materials and Methods

MATERIAL AND METHODS

The present *ex-vivo* study was carried out in the Department of Conservative Dentistry and Endodontics, Ragas Dental College and Hospital, Chennai and King Institute of Preventive Medicine & Research, Guindy.

ARMAMENTARIUM:

1. Electric pulp tester, Digitest™ II Pulp Vitality Tester (figure 1)
2. Gutta percha stick (figure 1)
3. X-ray unit-6010E , Adhitiya medical systems Ltd., India (figure 2)
4. Radiovisiography , EZ Dent-I Imaging software., Vatech (figure 2)
5. Local anaesthesia, Lignox 2% with adrenalin 1:80,000 (figure 1)
6. 2.5ml disposable syringe
7. Rubber dam kit, GDC®, UK (figure 1)
8. High speed airotor hand piece, NSK, Japan (figure 1)
9. Endo access bur, Dentsply Maillefer, Switzerland (figure 1)
10. ISO size #10,15 k-file stainless steel hand files, Mani Inc.,Japan (figure 1)
11. ISO size #15 H-file, Dentsply Maillefer, Ballaigus, Switzerland (figure 1)
12. ISO size #15, #20 Broaches, Pfiffer Dent, France (figure 1)
13. Diamond rotary disc, Dentsply Maillefer, Switzerland (figure 1)
14. 5.25% sodiumhypochlorite, Prime Dental (figure 1)

15. Normal saline, Eurolife Healthcare Pvt. Ltd (figure 1)
16. Eppendorf Tubes (figure 5)
17. 99% Ethanol (figure 9)
18. -80ºC deep freezer, Forma lab freezer ,Thermo Scientific™, US (figure 6)
19. QIAamp RNA extraction Kit, Qiagen (figure 7)
20. Micro Centrifuge tubes (figure 11)
21. Micropipette, Thermo Scientific™, US (figure 8)
22. Vortex Machine, VELP Scientific, Inc.,Italy (figure 14)
23. Centrifuge, Heraeus™ Pico™ 17 Microcentrifuge (figure 15)
24. NanoDrop™ 2000c Spectrophotometer, Thermo Scientific™, US (figure 17)
25. RevertAid First Strand cDNA Synthesis kit , Thermo Scientific™, US (figure 19)
26. Thermocycler, Veriti Thermal Cycler, Applied Biosystems™ (figure 22)
27. Real Time PCR, Roche LightCycler ® 480 Real-Time PCR System, Mannheim,Germany (figure 24)
28. Forward and reverse primers for leptin and cyclophilin (figure 25)
29. Power SYBR™ Green PCR master mix, Thermo Scientific™, US
30. Roche LightCycler ® 480 PCR plates (figure 26)

METHOD OF DATA COLLECTION:

Human dental pulp samples were collected from a total of thirty mandibular molars of healthy human subjects aged between 17 and 50 years which met the inclusion and exclusion criteria given below. The study was carried out with the understanding and written consent of each subject to donate their pulp tissue. The protocol was approved by the Institutional and Review Board (IRB) and ethical committee of Ragas Dental College, Chennai (Date: 29th Feb 2016)

INCLUSION CRITERIA:

- Patients of age group : 17 – 50 years (under no medication)
- Normal weight individuals (based on body mass index BMI) were included for the study.

EXCLUSION CRITERIA:

1. Patients with
    - diabetes
    - congenital leptin deficiency
    - lipodystropy
    - hypothalamic amenorrhea
2. Patients under
    - medications
    - chronic medical history

3. Patients who are underweight and overweight (based on body mass index BMI) were excluded.

STUDY GROUPS:

The teeth were divided into three groups (n=30)

Group 1: Acute pulpal pathosis (symptomatic irreversible pulpitis) (n=10)

Group 2: Chronic pulpal pathosis (asymptomatic irreversible pulpitis) (n=10)

Group 3: Healthy pulp (n=10)

SELECTION OF GROUPS:

All the thirty teeth were verified by pulp sensibility testing, Digitest™ II Pulp Vitality Tester (figure1) and digital radiographs in EZ Dent-I Imaging software (figure 2) along with the clinical history of signs and symptoms to access the extent of caries and the periapical changes

Group 1 **(n=10)** consisted of pulp tissue collected from ten mandibular molar teeth with clinical diagnosis of acute irreversible pulpitis (symptomatic). Diagnosis was made on the following criteria: patient had a recent history of severe spontaneous pain, with prolonged and lingering episodes of pain to thermal or cold stimuli, along with the clinical and radiographic examination determining the presence of near or actual pulp exposure, with or without widening of periodontal ligament.

Group 2 **(n=10)** contained pulp tissue collected from ten mandibular molar teeth with clinical diagnosis of chronic irreversible pulpitis

(asymptomatic). Teeth were placed into this group based on the following criteria: patient does not complain of any symptoms at present and gives a history of few episodes of intermittent or dull pain over the past one year. Diagnosis was based on the accidental clinical and radiographic examination which revealed a deep caries near or actual pulp exposure and with/without widening of periodontal ligament and presented with lingering pain to pulp sensibility testing .Three radiographs among the ten cases revealed periapical lesion.

Group 3 **(n=10)** consisted of healthy dental pulp tissue collected from ten teeth which included impacted, ectopically erupted mandibular molars. Teeth were selected based on the following criteria: verbal history which confirms no history of pulpal pain along with the clinical and radiographic examination which assures that these teeth had no caries, restorations, or periodontal disease.

SAMPLE COLLECTION:

GROUPS 1 and 2 (n=10 for each group):

The selected mandibular molar tooth were isolated with rubber dam, GDC®, UK (figure1) and access opening was done under local anaesthesia, Lignox 2% with adrenalin 1:80,000 (figure 1). After access opening, glide path was created using #10, #15-K file,Mani Inc.,Japan (figure 1) . Barbed broach #15 or ISO size #15 H-file, Dentsply, Switzerland (figure 3) is inserted

into the canal to extirpate the pulp tissue which is placed immediately in eppendorf tubes containing ethanol (figure 5).

Group 3 (n=10):

The extracted teeth were washed with 5.25% sodium hypochlorite (figure 1) after extraction to exclude the remnants of periodontal ligament that could contaminate the pulp tissue samples. The teeth were then immediately sectioned axially at the cemento-enamel junction (figure 4) by using a diamond rotary disc in a dental straight handpiece irrigated with saline solution. The pulp tissue was collected by using an endodontic broach #15 or ISO size #15 H-file, Dentsply, Switzerland and placed in the labeled Eppendorf tube containing ethanol immediately (figure 5).

SAMPLE STORAGE:

All the samples were stored at -80ºC Forma lab freezer, Thermo Scientific™ (figure 6) in the labeled Eppendorf tubes containing ethanol till further analysis.

PROCEEDURE:

The pulp tissue samples were taken from -80°C deep freezer and placed aside for thawing till they reach room temperature

PROTOCOL FOR THE EXPERIMENT :

1. Total RNA extraction from the tissue sample
2. RNA quantification
3. Synthesis of cDNA
4. Relative quantification with RT PCR machine
5. Interpretation of the RT PCR results and graphs

A) Extraction of RNA from the tissue sample:

Total RNA extraction from human dental pulp tissue samples were done by using QIAamp® RNA extraction Kit, Qiagen (figure 7)

The following materials were provided in the kit

S.NO	Kit Components	Quantity(for50 preparations)
1	Lysis buffer (AVL)	31ml
2	Ellusion buffer (AVE)	3*2 ml
3	Wash buffer conc. (AW1)	19ml
4	Wash buffer conc. (AW2)	13ml
5	Carrier RNA (poly A)	310 µl
6	QIAamp Mini Spin Columns	50
7	Micro Centrifuge tubes	1.5ml
8	Collection tubes (2 ml)	200

Miscellaneous: Pipette (1000µl, 100µl), disposable sterile tips, tissue papers, 99% Ethanol

PREPARATION OF BUFFERS: (figure 9)

Lysis buffer (AVL):

310µl of AVE buffer is pipetted out to one tube of lyophilized carrier RNA (310µg) and thoroughly dissolved. Aliquot 28µl and store at -20°C. Aliquot 560µl of AVL buffer and is stored at room temperature. AVL buffer is always freshly prepared. 5.6µl of AVE+ Carrier RNA (kept at -20°C) is added to 560µl of AVL buffer (kept at room temperature).

Wash buffer 1 (AW1):

Buffer AW1 is supplied as a concentrate. Appropriate amount of ethanol is added as indicated on the bottle before using for the first time and stored at room temperature.

Wash buffer 2 (AW2):

Buffer AW2 is also supplied as a concentrate. Appropriate amount of ethanol is added as indicated on the bottle before using for the first time and stored at room temperature.

RNA EXTRACTION PROCEDURE:

1. 560µl of prepared Buffer AVL containing carrier RNA was pipetted into 1.5ml micro centrifuge tube and the tissue samples were added into the tube (figure 10, 11).
2. The samples are mechanically disturbed using sterile stick (figure 12,13) and incubated at room temperature for 10 min and then mixed by pulse vortexing for about 15sec .
3. Tubes were briefly centrifuged to remove drops from the inside of the lid.
4. 560µl of ethanol was added to the sample and mixed immediately by pulse vortexing for 15 sec (figure 14) and centrifuged briefly.
5. 630µl of the solution prepared in last step is carefully applied to the QIAamp Mini column (figure 16) and centrifuged at 8000 rpm for 1 min. The column is placed into a clean 2ml collection tube and the tube containing the filtrate is discarded.
6. The last step is repeated again since the sample volume is greater than 140 µl.
7. 500µl of Buffer AW1 is added and centrifuged at 8000 rpm for 1 minute. Place column into a clean 2ml collection tube and the tube containing the filtrate is discarded.
8. 500µl of Buffer AW2 is then added and centrifuged at full speed (14000rpm) for 4 minutes (figure 15).

9. Again the column is placed in a clean 2ml collection tube and the old collection tube with the filtrate is discarded and centrifuged at full speed for 1 minute.
10. The column is placed in a clean 1.5ml micro centrifuge tube. 60µl of Buffer AVE is added, incubate at room temperature for 1 minute and centrifuged at 8000rpm for 1 minute.
11. RNA is stored at -70°C till further use.

B) RNA quantification:

The extracted Total RNA was quantified by using the NanoDrop™ 2000c Spectrophotometer, Thermo Scientific™, US (figure 17) and the purity of RNA was assessed (figure 18).

C) Synthesis of cDNA :

The total tissue RNA was reverse transcribed to complementary DNA (cDNA) using RevertAid First Strand cDNA Synthesis kit ,Thermo Scientific™, US (figure 19)

PROCEEDURE FOR REVERSE TRANSCRIPTION:

1. A master mix has to be prepared for the number of PCR reactions to be carried out.

S.No	Component	Quantity (for 1 reaction i.e. 1x)
1	5X Reaction Buffer	4 µL
2	RiboLock RNase Inhibitor	1 µL
3	10mM dNTP Mix	2 µL
4	RevertAid Reverse Transcriptase	1 µL
5	Random Hexamer Primer	1 µL
6	Nuclease-free Water	11 µL
	Total	20 µL

2. 5µl of the extracted total RNA was added to the Master mix.
3. The components are mixed, briefly centrifuged and pipetted carefully into the wells of the thermocycling plates (figure 20,21)
4. The plates are incubated in the thermocycler, programmed as per manufacturer's recommendation (figure 22, 23)

Initial step - 5 min at 25°C

Reverse Transcriptase step - 60 min 42°C

Inactivation of RT enzyme - 5 min 70°C

The reverse transcription reaction product can be directly used in qPCR applications or stored at -20°C for less than one week.

D) Relative quantification with RT PCR :

Relative quantification was done in Roche, LightCycler® 480,Germany Real Time-PCR and RT-PCR master mix reagent kit is obtained from Power SYBR Green PCR master mix, Applied Biosystems™

1. The following reagents are to be prepared separately for forward and reverse primers of leptin and cyclophilin respectively in 1.5 ml reaction tube. Leptin is the target gene and Cyclophilin is the house keeping gene in our study (figure 25)

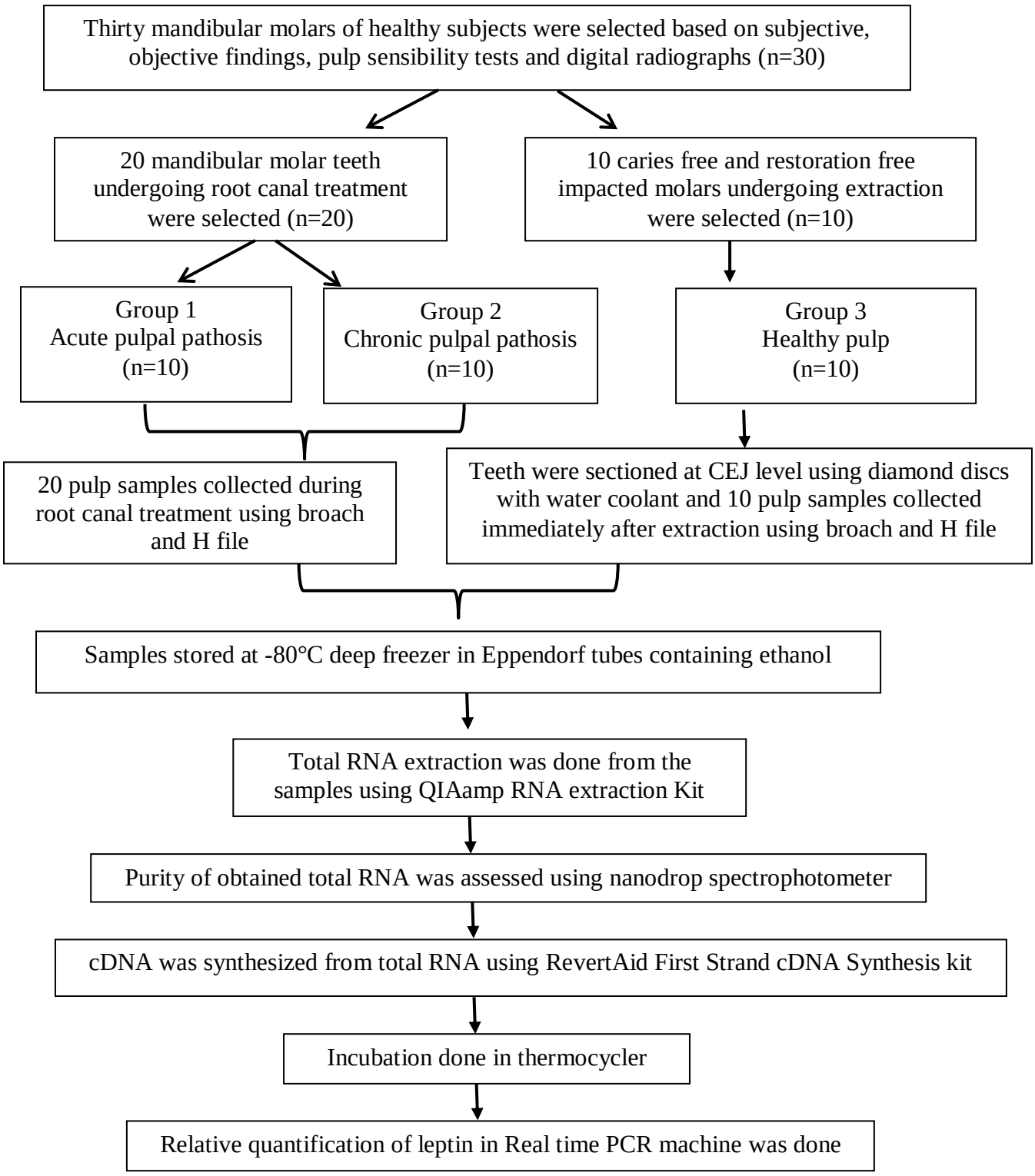
S.No	Component	Quantity
1	Power SYBR Green PCR master mix	12.5 µL
2	Forward Primer	1.0 µL
3	Reverse Primer	1.0 µL
4	Nuclease free water	8.5 µL
	Total	23 µL

2. 2 µL of cDNA template is pipetted out into the mixed reagent and briefly centrifuged.
3. All the reactions were carried out in triplicate

4. The reagent mix is pipetted into the wells of the LightCycler® 480 Multiwell Plate and the plate is sealed properly with a LightCycler® 480 Sealing Foil (figure 26)
5. LightCycler® 480 Software is started and the thermal-cycling conditions are programmed for Power SYBR Green PCR master mix . the temperature cycles followed were – preheating at 50 °C for 2 min, heating at 95 °C for 10 min. Eventually 41 amplification cycles were carried out under the following conditions - denaturation for 15 s at 95 °C , annealing for 1 min and extension at 58 °C (Martin-Gonzalez 2009).[72]
6. The threshold cycle (CT) of the samples in each well was determined by the LightCycler® 480 real time software by ΔΔCT-Method and amplification curves were generated using CT values of each samples (figure 27,28,29,30,31).

Hence in summary, total RNA extraction from were done from the pulp tissue samples using Qiagen kit. Complementary cDNA was synthesized using random primers and Real time PCR (Roche, LightCycler® 480,Germany) run was performed for relative quantification and gene expression of leptin in pulp tissue samples .

METHODOLOGY FLOWCHART



Figures

FIGURE 1: ARMAMENTARIUM REQUIRED

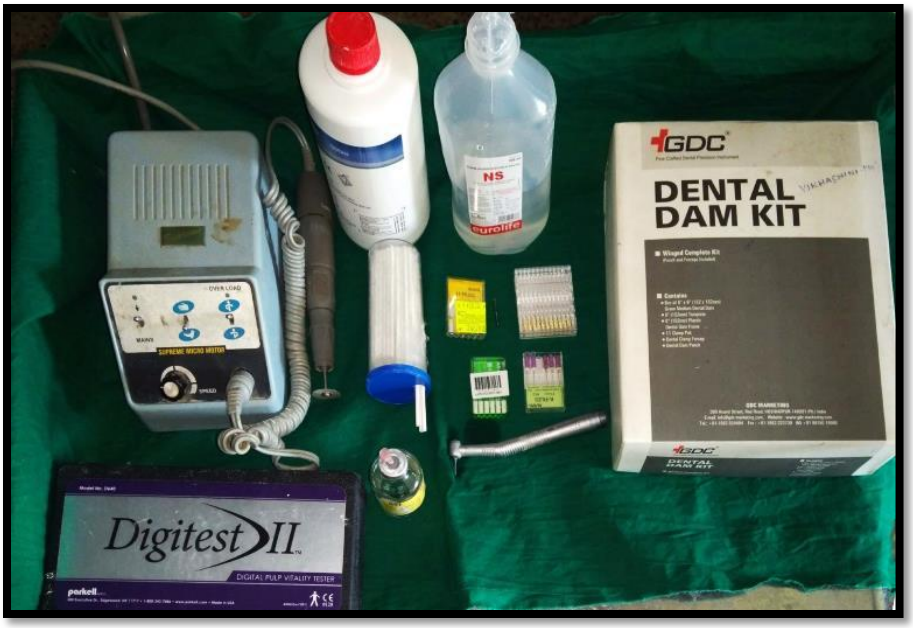


FIGURE 2: X- RAY UNIT - 6010E AND RVG EZ DENT-I IMAGING SOFTWARE

FIGURE 3: COLLECTION OF PULP TISUE SAMPLES USING BROACH ISO SIZE #20

FIGURE 4: DECORONATION OF EXTRACTED TOOTH

FIGURE 5: SAMPLES IN EPPENDORF TUBES

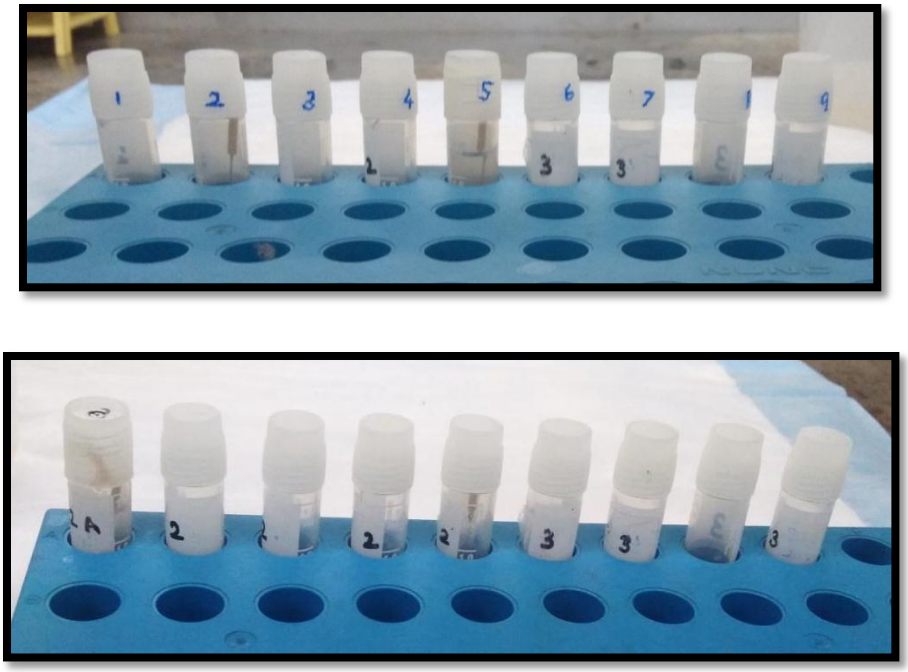


FIGURE 6: DEEP FREEZER AT -80ºC (THERMO SCIENTIFIC™, US)

FIGURE 7: QIAamp RNA EXTRACTION KIT, QIAGEN

FIGURE 8: MICROPIPETTE, THERMO SCIENTIFIC™, US

FIGURE 9: PREPARATIONS OF BUFFERS

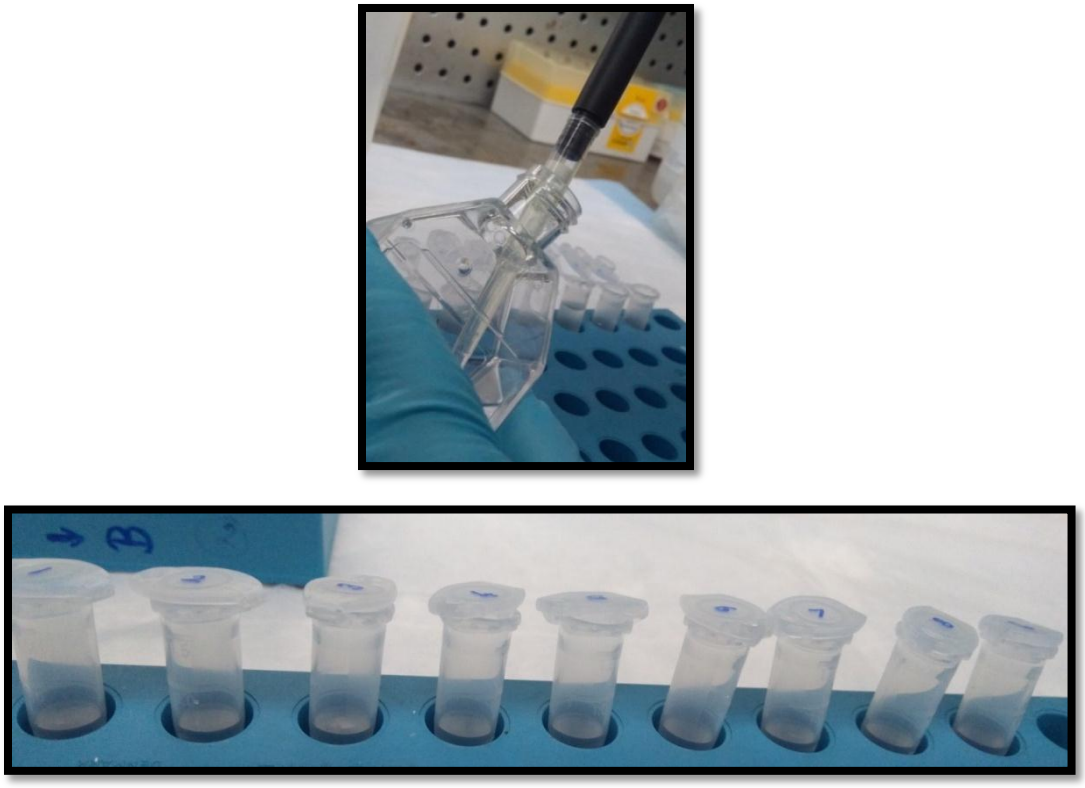


FIGURE 10: PULP SAMPLE TAKEN OUT OF EPPENDORF TUBES

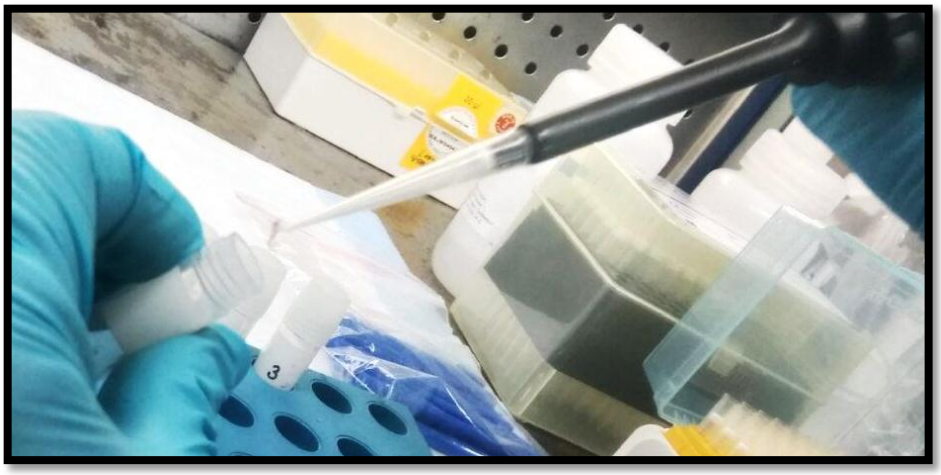


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FIGURE 11: ADDITION OF PULP SAMPLE INSIDE THE TUBE CONTAINING BUFFERS

FIGURE 12: MECHANICALLY DISTURBING PULP TISSUE USING STERILE STICK

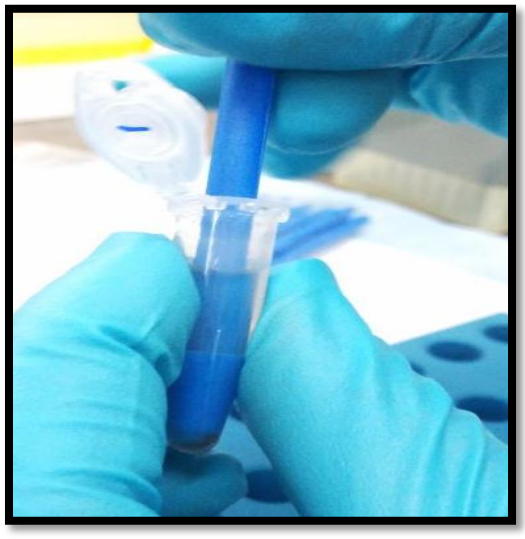


FIGURE 13: HOMOGENIZED SOLUTION

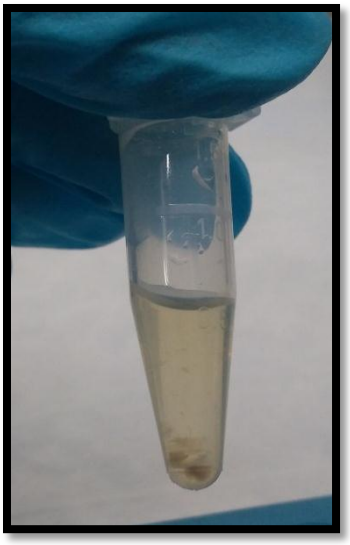
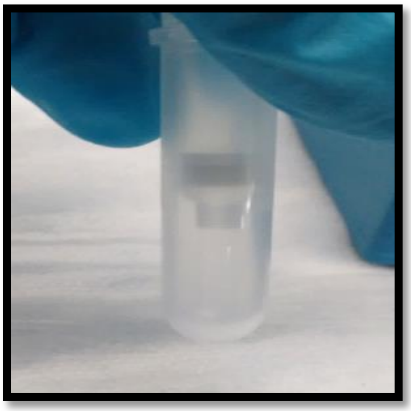


FIGURE 14: MIXING IN VORTEX MACHINE (VELP SCIENTIFIC, ITALY)

FIGURE 15: CENTRIFUGATION DONE IN PICO™ 17 MICROCENTRIFUGE

FIGURE 16: QIAAMP MINI SPIN COLUMNS



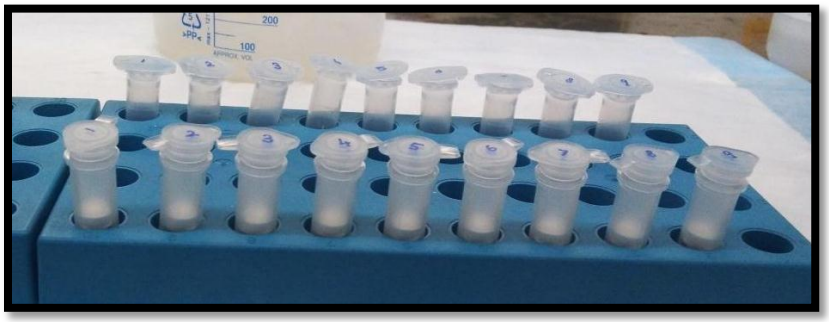


FIGURE 17: ASSESSING THE PURITY OF RNA USING NANODROP™ 2000C SPECTROPHOTOMETER (THERMO SCIENTIFIC™, US)

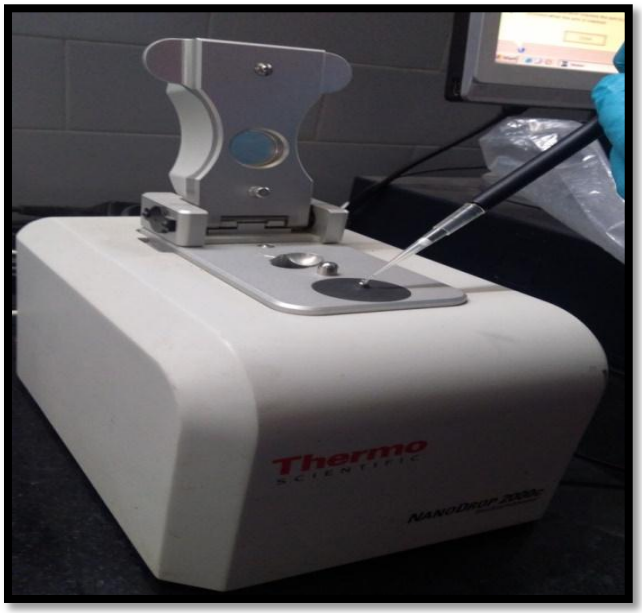


FIGURE 18: CONCENTRATION OF RNA DISPLAYED ON MONITOR

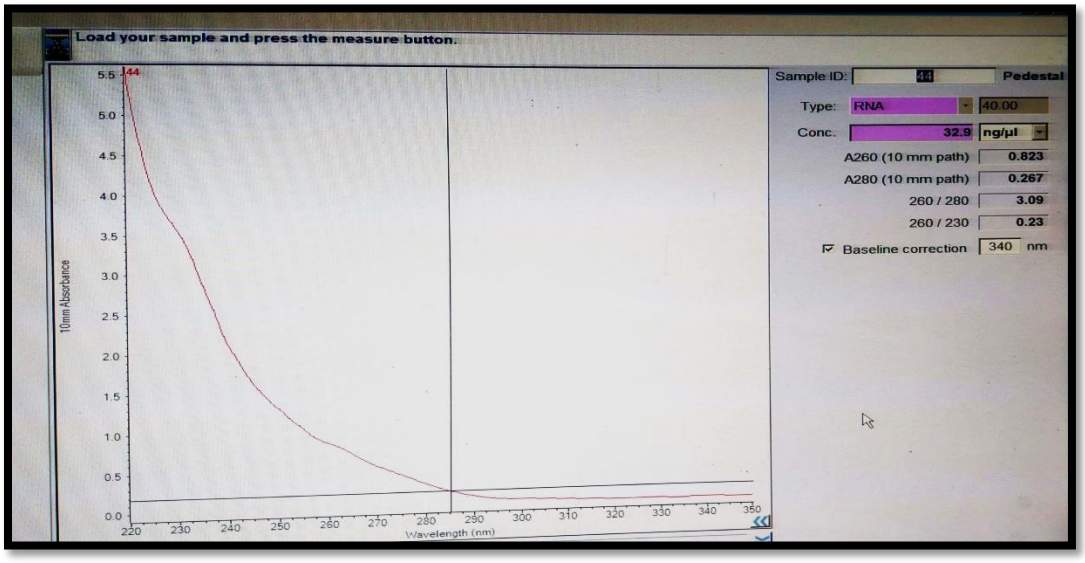
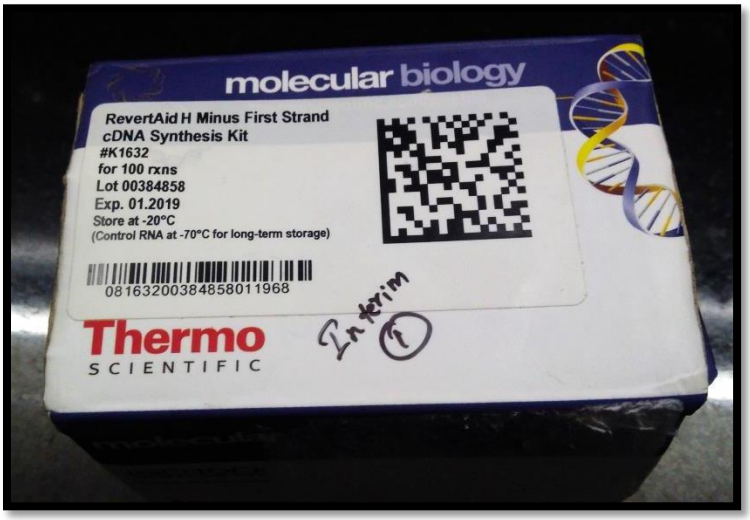


FIGURE 19: COMPONENTS OF REVERT AID FIRST STRAND CDNA SYNTHESIS KIT (THERMO SCIENTIFIC™, US)



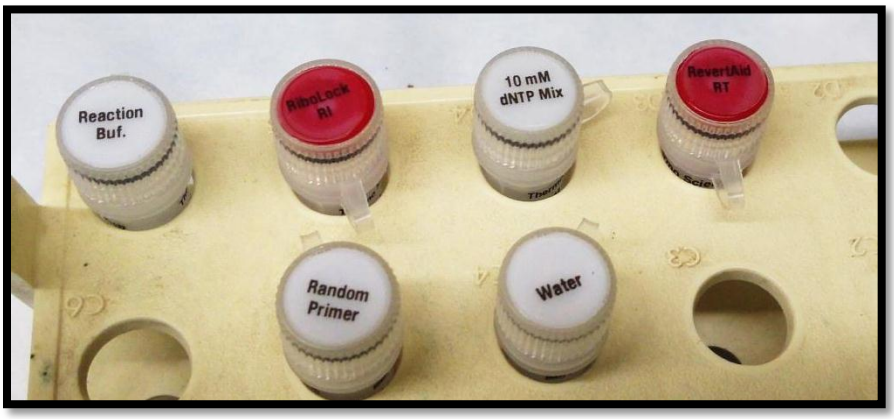


FIGURE 20: ADDITION OF PREPARED MIX USING RANDOM PRIMERS INTO THE PLATE

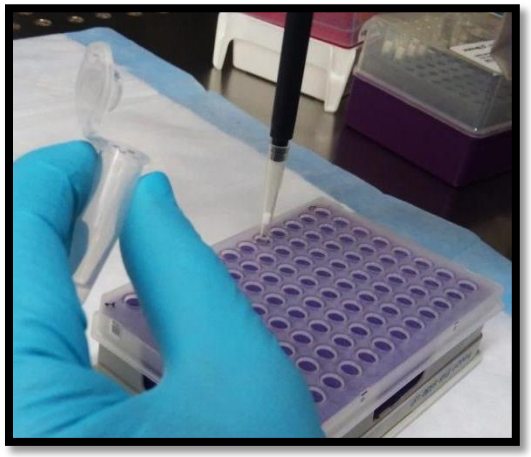
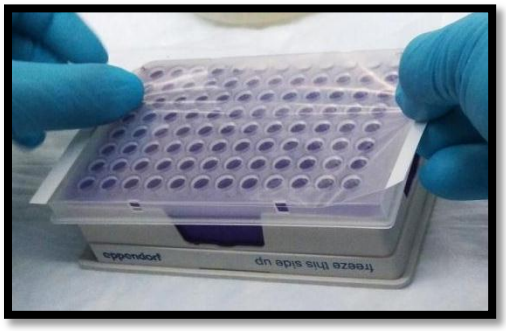


FIGURE 21: SEALED PLATE



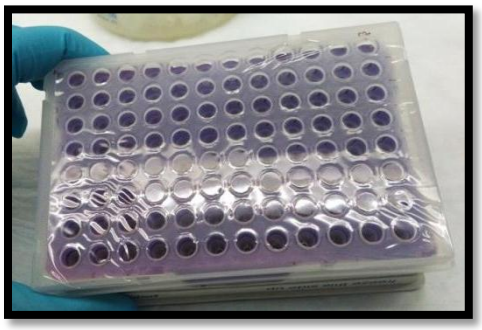


FIGURE 22: PLACING PLATES INSIDE THERMOCYCLER (VERITI THERMAL CYCLER, APPLIED BIOSYSTEMS™)

FIGURE 23: MANUALLY SETTING UP THE THERMAL CONDITIONS IN THERMOCYCLER

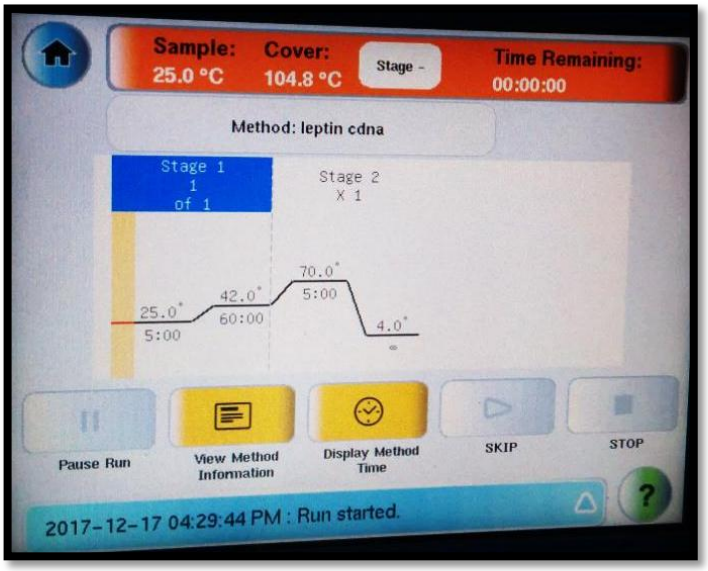


FIGURE 24: REAL TIME PCR MACHINE (LIGHT CYCLER® 480, ROCHE, GERMANY)

FIGURE 25: FORWARD AND REVERSE PRIMERS FOR LEPTIN AND CYCLOPHILIN

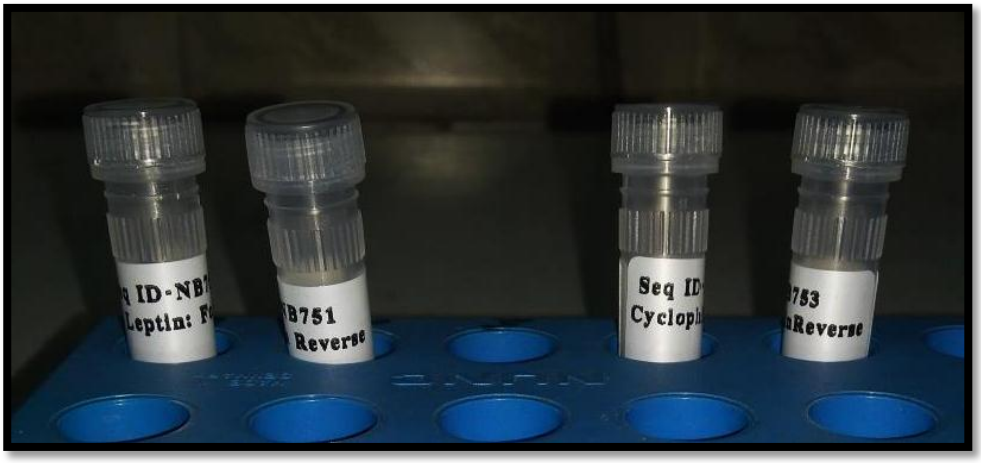


FIGURE 26: ROCHE LIGHTCYCLER® 480 MULTIWELL PCR PLATE, GERMANY

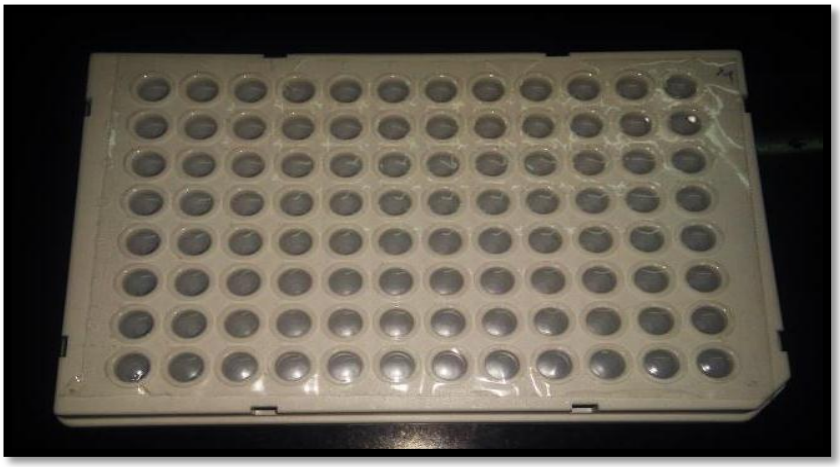


FIGURE 27: AMPLIFICATION CURVE DISPLAYED AFTER THE COMPLETION OF CYCLES EXPRESSING BOTH LEPTIN AND CYCLOPHILIN

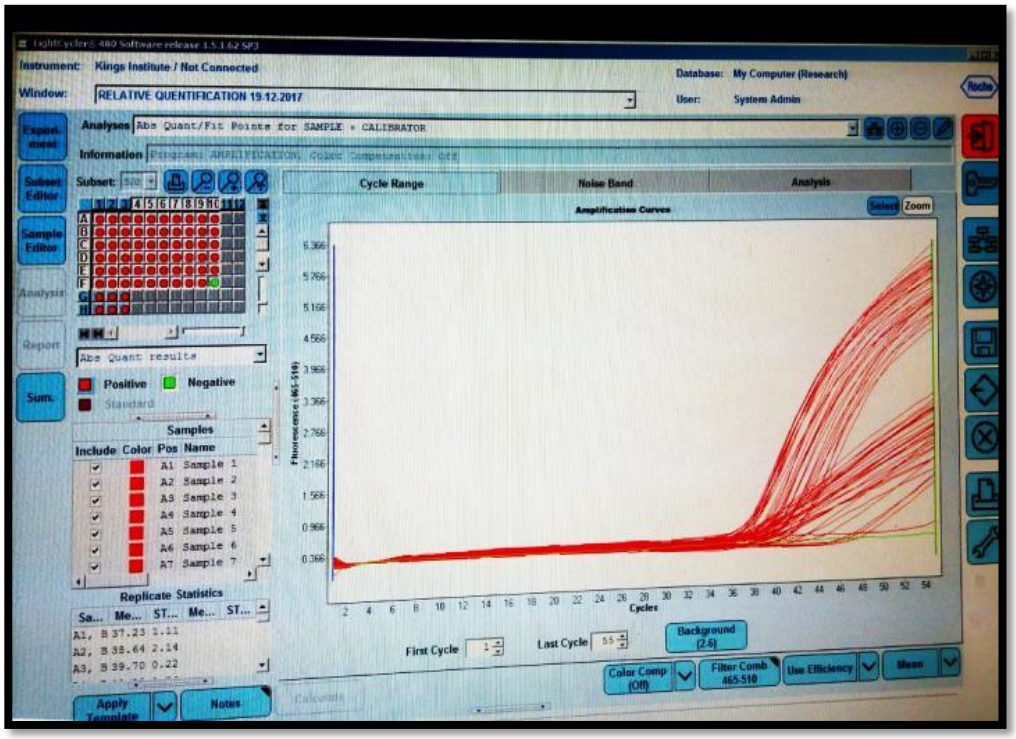


FIGURE 28: AMPLIFICATION CURVE OBTAINED IN REAL TIME PCR FOR LEPTIN IN ACUTE AND CHRONIC SAMPLES

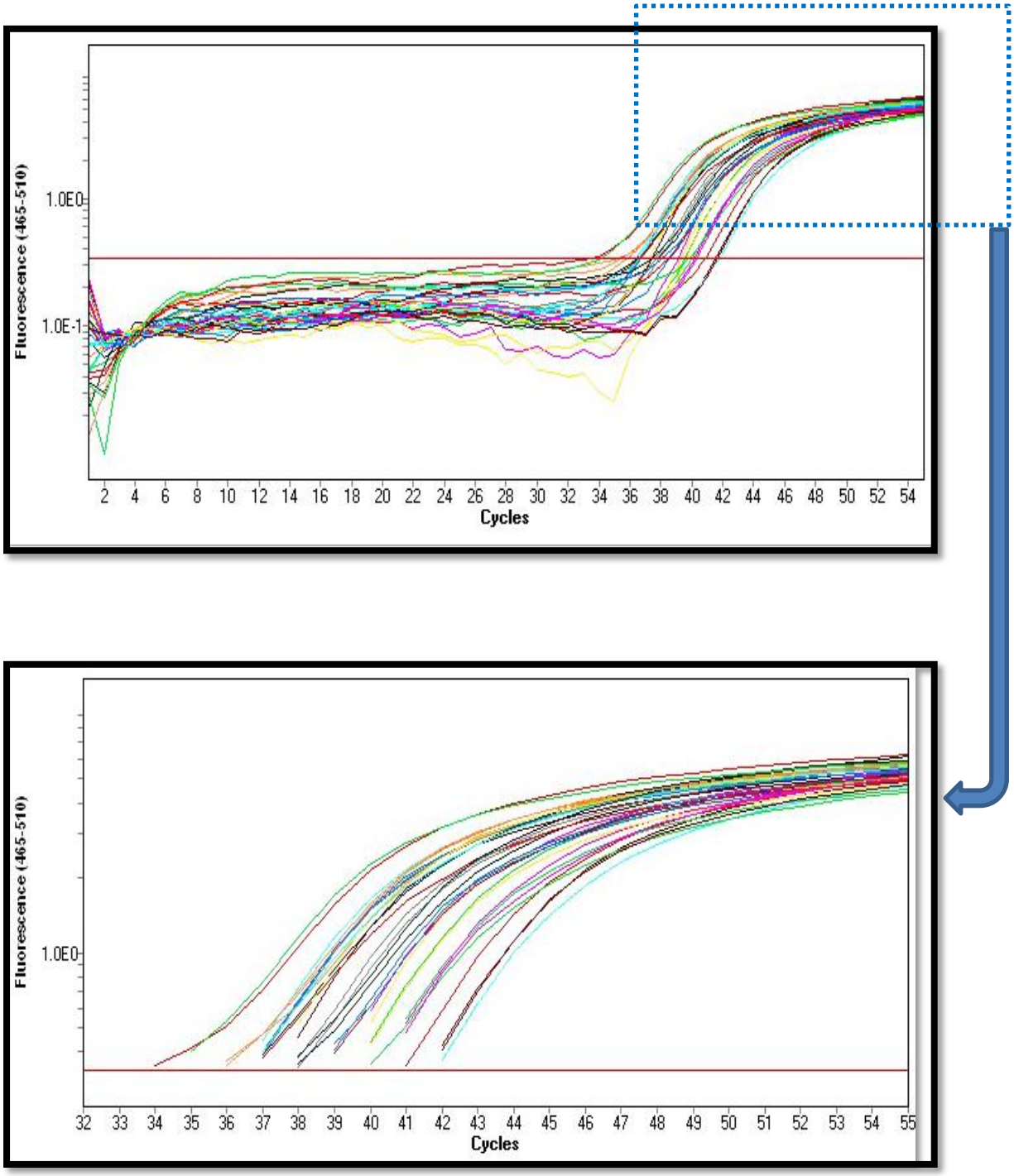


FIGURE 29: AMPLIFICATION CURVE OBTAINED IN REAL TIME PCR FOR LEPTIN IN HEALTHY PULP TISSUE SAMPLES

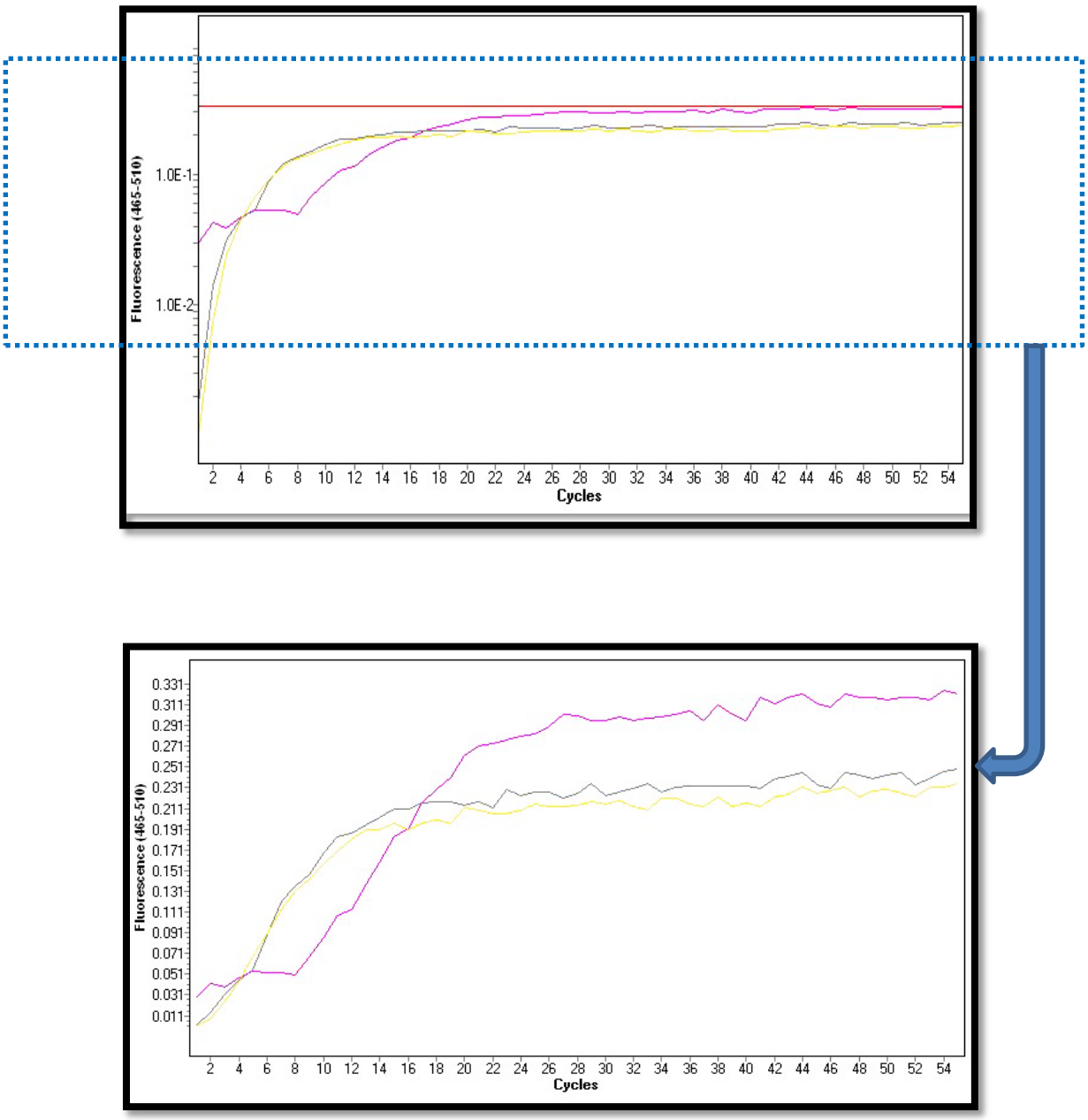


FIGURE 30: AMPLIFICATION CURVE OBTAINED IN REAL TIME PCR FOR CYCLOPHILIN IN ACUTE AND CHRONIC PULP TISSUE SAMPLES

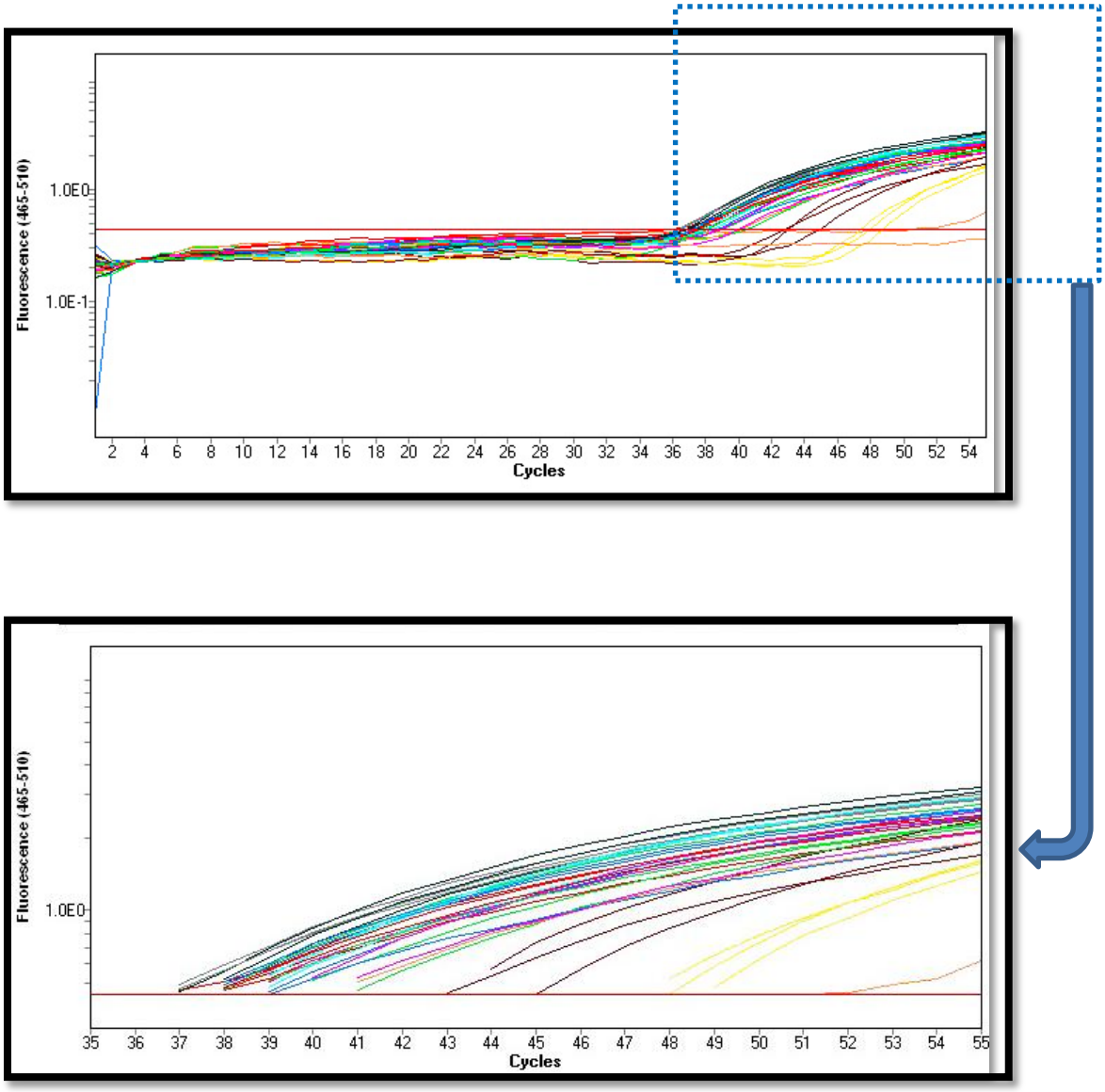
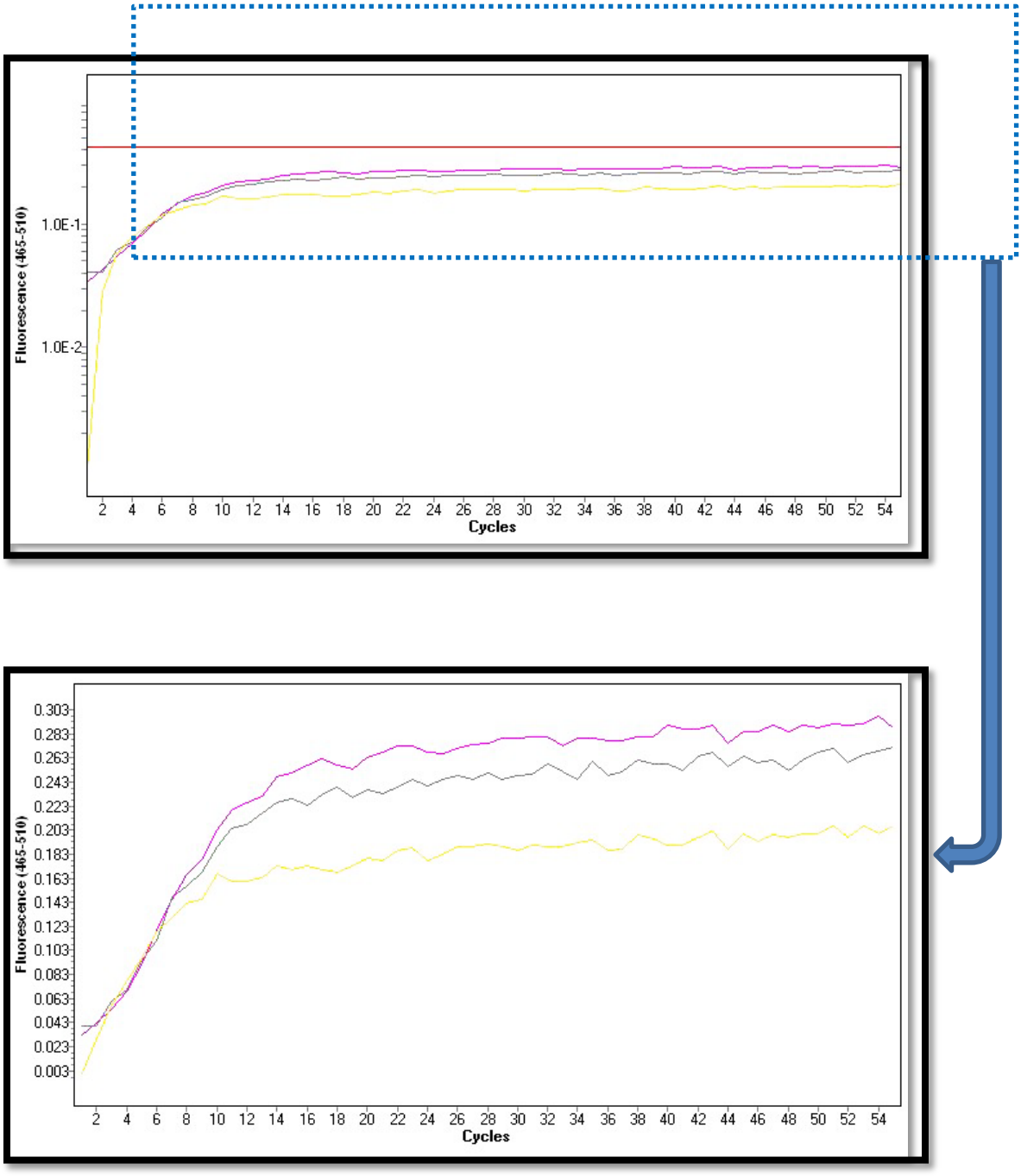


FIGURE 31: AMPLIFICATION CURVE OBTAINED IN REAL TIME PCR FOR CYCLOPHILIN IN HEALTHY PULP TISSUE SAMPLES



Results

RESULTS

This study was designed to evaluate the expression of leptin in human dental pulp samples collected from healthy, acute and chronic inflammatory conditions of pulpal pathosis. Relative quantification of leptin was performed by real time quantitative polymerase chain reaction machine (Roche, Light Cycler® 480 Real Time-PCR, Germany). Leptin expression was normalized to internal control Cyclophilin. The threshold cycle for each template was analyzed by Light Cycler® 480 Software and the data were expressed as crossing point values and amplification curves for each group. The pulp tissue samples were collected from a total of 30 patients divided into three groups

Group 1- Acute pulpal pathosis

Group 2- Chronic pulpal pathosis

Group 3- Healthy pulp

Data obtained were compiled in Microsoft Excel spreadsheet systematically. Statistical analysis was performed using Statistical Package for Social Sciences software (SPSS version 22, USA). The confidence interval was set at 95% and p value was set for 0.05 and any value equal to or less than 0.05 was considered to be significant. One- way ANOVA test was used to compare the leptin and cyclophilin expression among the three study groups. Post HOC TURKEY tests were used for pair wise multiple comparisons to identify if any significant difference in leptin expression exists among the study groups.

All the reactions were run in triplicate. Table 1 and graph 1shows the descriptive statistics for the distribution of Cp values for leptin gene expression in acute pulp tissue samples. The mean Cp for leptin gene in group I was 39.854 with a standard deviation of 2.469.

Table 2 and graph 2 shows the descriptive statistics for the distribution of Cp values for leptin gene expression in chronic pulp tissue samples. The mean Cp for leptin gene in group 2 was 37.878 with a standard deviation of 1.498

Table 3and graph 3 shows the descriptive statistics for the distribution of Cp values for leptin gene expression in healthy pulp tissue samples. The mean Cp for leptin gene in group 3 was 33.302 with a standard deviation of 2.260

Descriptive statistics along with the mean and standard deviation for acute, chronic and healthy pulp tissue samples for leptin has been given in table 4, graph 4. The results showed that the highest expression of leptin is detected in acute (GROUP 1) pulp tissue samples with mean and standard deviations 39.854 ± 2.469; followed by chronic (GROUP 2) pulp tissue samples with mean and standard deviations 37.878 ± 1.498 and healthy pulp tissue samples (GROUP 3) with mean and standard deviations 33.302 ± 2.260. While comparing the mean and standard deviations of the study groups for the expression of leptin, there was statistically significant difference between GROUP 1, GROUP 2 and GROUP 3 with a p value of 0.000 ($p < 0.05$).

The intergroup comparison of expression of leptin between the study groups has been given in table 5, graph5. The results showed that there was statistically significant difference between acute and healthy groups with p value of 0.000 ($p < 0.05$). While comparing chronic and healthy groups, there was significant difference between the groups with p value of 0.000 ($p < 0.05$). On comparison between acute and chronic groups, there was statistically significant difference between acute and healthy groups with p value of 0.001 ($p < 0.05$).

Table 6 and graph 6 shows the descriptive statistics with their mean and standard deviation for the distribution of Cp values for internal control Cyclophilin gene expression in acute, chronic and healthy pulp tissue samples. The results showed that the highest expression of leptin is detected in acute (GROUP 1) pulp tissue samples with mean and standard deviations 40.439 ±3.618; followed by chronic (GROUP 2) pulp tissue samples with mean and standard deviations 38.148000 ± 1.189 and healthy (GROUP 3) pulp tissue samples with mean and standard deviations 37.438± 0.995.

While comparing the mean and standard deviations of the study groups for the expression of the housekeeping gene Cyclophilin, there was no statistically significant difference between GROUP 1, GROUP 2 and GROUP 3 with a p value of 0.230 ($p >0.05$) (Graph 7).

Tables and Graphs

Table 1: Descriptive statistics of Cp values for leptin in Acute pulp tissue samples (Group 1)

S.NO	Cp values
1	38.67
2	40.23
3	38.29
4	40.13
5	39.96
6	41.23
7	40.90
8	38.63
9	39.79
10	40.67
Mean ±SD	39.854 ±2.469

Graph 1: Descriptive statistics of Cp values for leptin in Acute pulp tissue samples (Group 1)

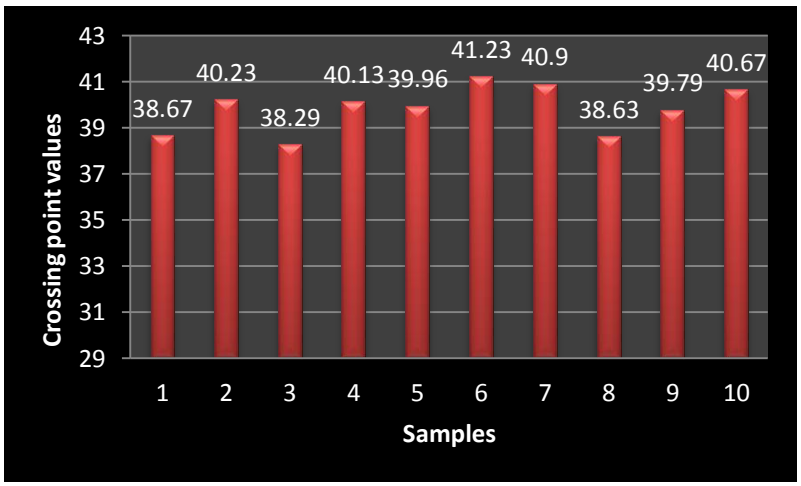


Table 2: Descriptive statistics of Cp values for leptin in Chronic pulp tissue samples (Group 2)

S.NO	Cp values
1	36.84
2	40.40
3	38.71
4	41.65
5	38.03
6	38.84
7	37.23
8	36.70
9	39.24
10	40.85
Mean ±SD	37.878±1.498

Graph 2: Descriptive statistics of Cp values for leptin in Chronic pulp tissue samples (Group 2)

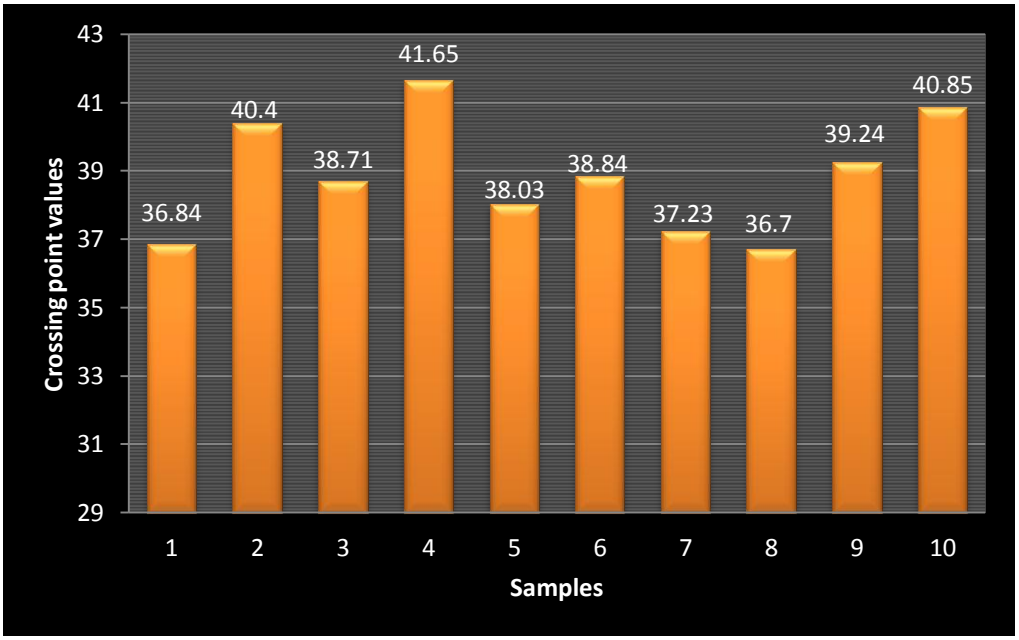


Table 3: Descriptive statistics of Cp values for leptin in Healthy pulp tissue samples (Group 3)

S.NO	Cp values
1	38.29
2	33.73
3	34.39
4	33.67
5	30.87
6	31.54
7	33.74
8	32.65
9	33.93
10	30.21
Mean ±SD	33.302±2.260

Table 3: Descriptive statistics of Cp values for leptin in Healthy pulp tissue samples (Group 3)

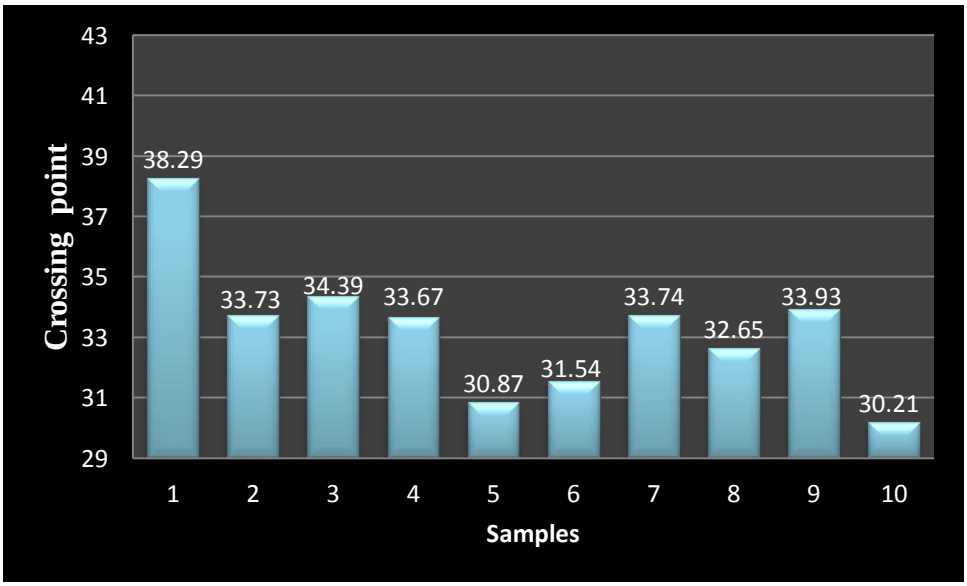


Table 4: Comparison of mean leptin expression among the study groups

Group	Mean ± SD	P value
Group I	39.854 ±2.469	0.000* (p<0.05)
Group II	37.878 ±1.498	
Group III	33.302 ± 2.260	

*Statistically Significant

Graph 4: Comparison of mean leptin expression among the study groups

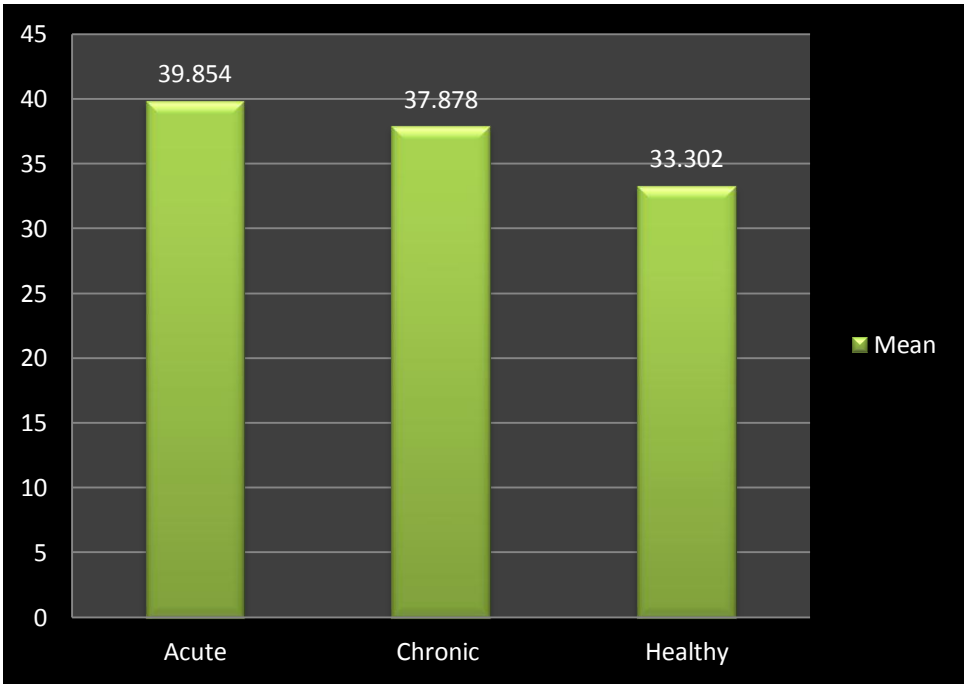


Table 5: Intergroup comparison of leptin expression among the study groups using Post Hoc turkey test

Group	Mean ± SD	P value	Significance
Group 1 and group 3	39.854 ± 2.469	0.000*	$p < 0.05$
Group 2 and Group 3	37.878 ± 1.498	0.000*	$p < 0.05$
Group 1 and Group 2	33.302 ± 2.260	0.001*	$p < 0.05$

*Statistically Significant

Graph 5: Intergroup comparison of leptin expression among the study groups using Post Hoc turkey test

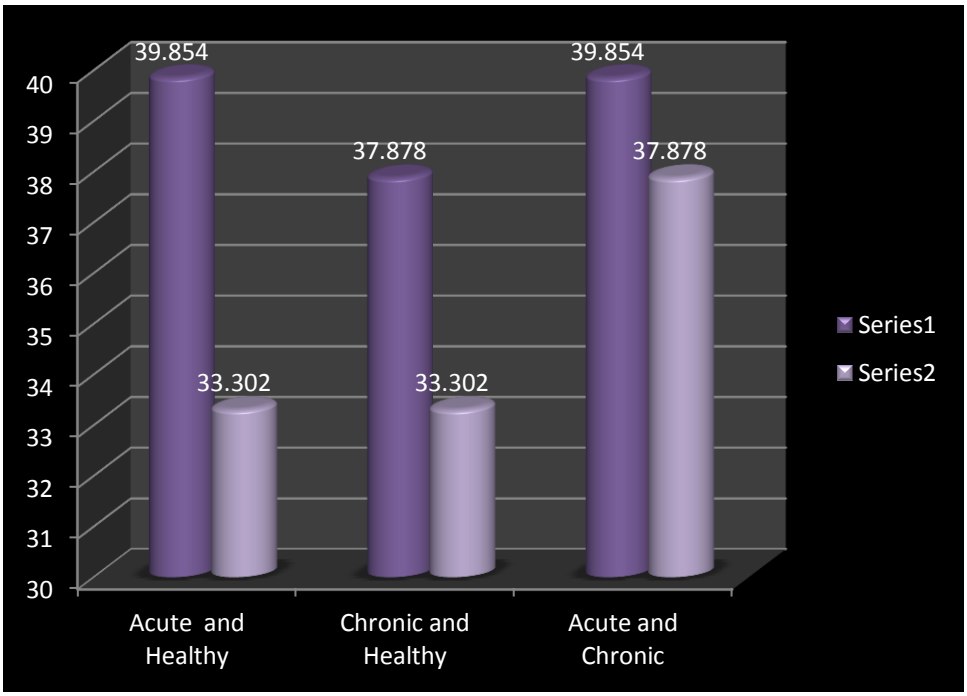


Table 6: Descriptive statistics of Cp values for cyclophilin expression among the study groups

Samples	Group 1	Group 2	Group 3
1	48.03333	38.406	37.25
2	43.65333	37.17667	37.54
3	38.30333	39.23667	38.19
4	37.19333	39.16	38.75
5	38.38333	36.57333	36.14
6	39.62	37.68	36.07
7	40.44667	38.38	38.86
8	37.65333	38.71	37.84
9	42.11667	38.48667	36.55
10	38.99	37.67	37.19
Mean ±SD	39.38± 3.618	38.148 ± 1.189	37.438±0.99

Graph 6: Descriptive statistics of Cp values for cyclophilin expression among the study groups

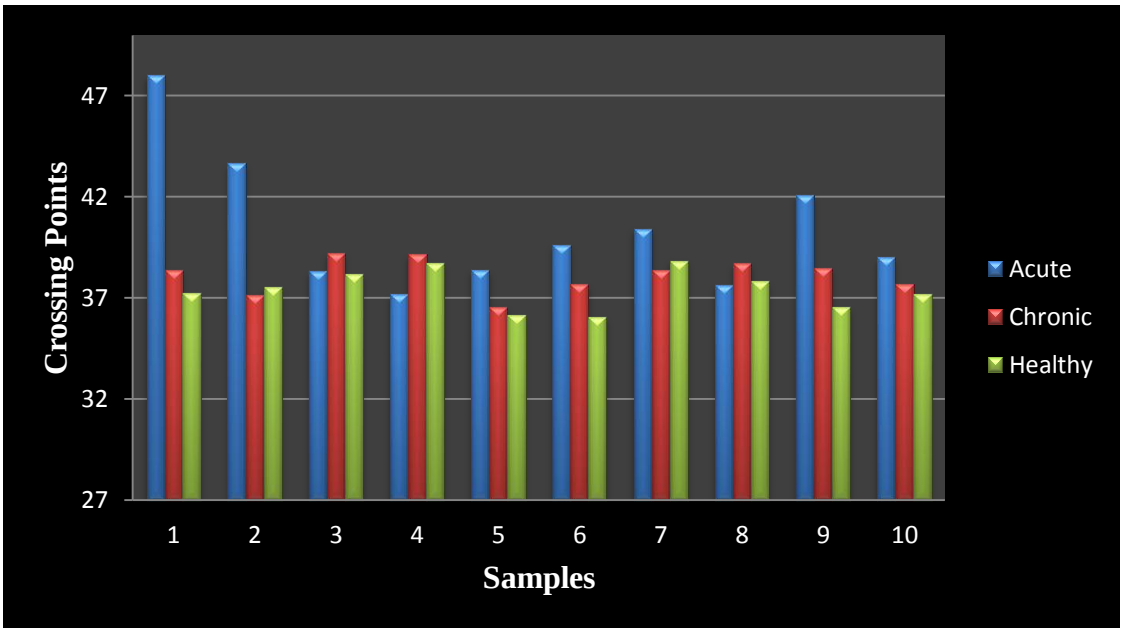
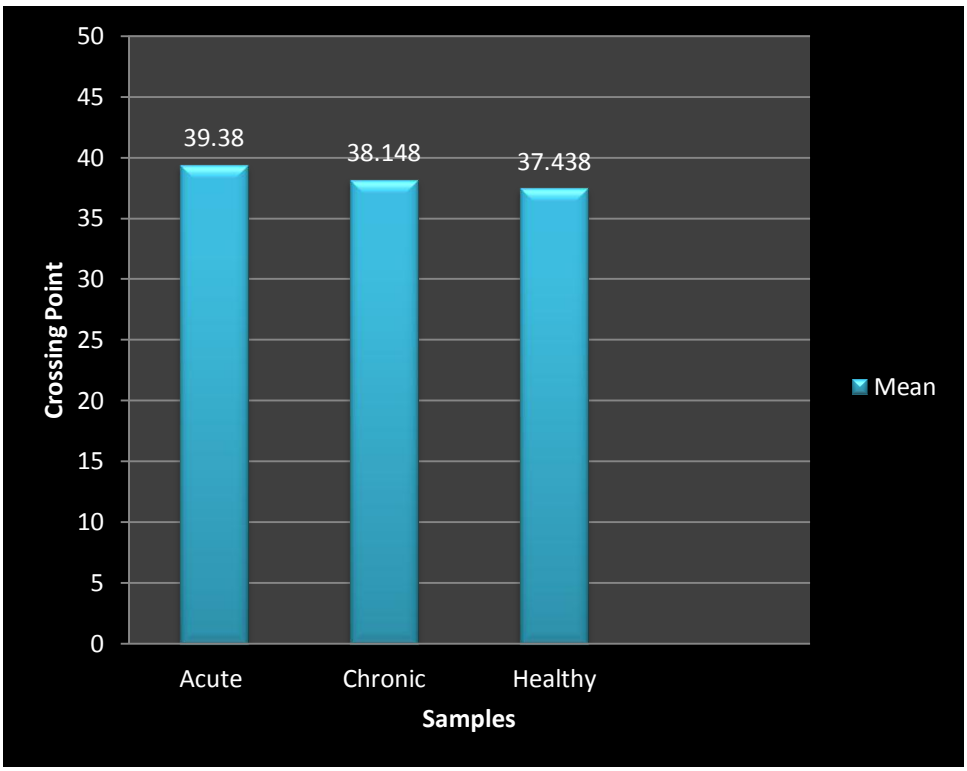


Table 7: Comparison of mean cyclophilin expression among the study groups

Group	Mean ± SD	P value
Group I	39.38 ± 3.618	0.230 (p>0.05)
Group II	38.148 ± 1.189	
Group III	37.438 ± 0.99	

Not Statistically Significant

Graph 7: Comparison of mean cyclophilin expression among the study groups



Discussion

DISCUSSION

Dental caries, a chronic infectious disease results from the progressive penetration of the oral microorganisms which proliferates and release acids into enamel, dentin and pulp resulting in demineralization of enamel and dentin. The proliferation and metabolic activity of these oral cariogenic bacteria leads to the release of bacterial metabolic by products and their cell wall components into dentinal tubules and their diffusion towards the peripheral pulp.[43] The dental pulp is a loose connective tissue containing diverse cell populations including odontoblasts, fibroblasts, ground substances, nerves, blood vessels, interstitial fluid and other cellular components. Recognition of these varied bacterial components by the host cells at the dentin-pulp interface by acting as antigens triggers multi cascades of host protective events including antibacterial, inflammatory and immune responses in turn affecting the gene expression in host. This specific reaction induced in a host following an antigen stimulus is known as immune response.[38]

Pulp inflammation i.e. pulpitis is a part of the fundamental protective immune response of the host to tissue damage. This inflammation usually dampens after the removal of caries lesion along with the infected pulp tissue containing microorganisms by the dental practitioner which is followed by the neutralization of diffused intratubular components by pulpal immune system. Depending upon the extent of tissue injury and the interaction of the host

tissue in an inflexible environment of the pulp chamber results in a broad spectrum of inflammatory and reparative events.[28]

Based on the specificity of the reaction, there are two kinds of immunological defenses: Natural or innate immunity and Cell mediated or Acquired immunity.

Upon initial invasion of microbes, innate immunity is activated. Innate immunity is not antigen-specific and provides immediate host response against pathogens. It recognizes the molecular patterns common to microbes by using receptors to initiate bacterial internalization and phagocytosis. In oral environment, the innate immune system includes epithelial barriers, circulating cells and proteins that slows down the bacterial invasion and eliminates them by recognizing microbes or substances produced by them.[42]

Adaptive immunity or acquired immunity is antigen specific immunity and it enhances the protective mechanisms of non-specific innate immunity. In contrast to innate immunity, the defense mechanisms in adaptive immunity specifically and selectively recognize and eliminate the invading pathogens and their by-products.[44]

Through the exquisite modulated interactions, the two types of immune regulatory mechanisms - innate and adaptive, work synergistically to exhibit more effective immunity. Dental pulp is a highly dynamic tissue that is decked with a network of resident immunocompetant cells. These immune cells are

present in both normal and pathologic pulp. In the normal pulp they are responsible for regulation of cellular density and are essential for the control of cellular proliferation and apoptosis, whereas in case of inflamed or exposed pulp they contribute to resolve the inflammatory process.[34]

All the cells of the immune system are derived from hematopoietic stem cells present in bone marrow and fetal liver. They differentiate either along lymphoid or myeloid pathway. Myeloid precursor cells lineage gives rise to mononuclear phagocytes - monocytes in blood, macrophages in tissues; polymorphs (basophils, eosinophil and neutrophils), dendritic cells, erythrocytes, platelets, mast cells. Lymphoid precursor differentiates into T (thymus-dependent) lymphocytes, B (bone marrow derived) lymphocytes and Natural Killer lymphocytes.[124]

The dental carious lesions usually progresses slowly into the dental pulp and hence the onset of innate immune response is difficult to specify in the dentin-pulp complex. *Pashley DH* in 1985 measured the dentin permeability by fluid filtration method and noted that there is a significant decrease in dentin permeability within few hours when fresh intact dentin was exposed peripherally.[87] Hence one of the initial protective response of the host to the invading bacteria and their by- products is the increased outward flow of dentinal fluid due to increased intra pulpal pressure followed by decreased dentinal permeability. This dynamic change blocks or reduces the permeation of noxious elements through dentinal tubules.[42]

Histological studies have shown that early carious lesion leads to morphological changes in odontoblasts and dental pulp.[76,77] Odontoblasts are the first pulpal cells to encounter the antigenic challenges as they are located at the pulp dentin interface along with their cellular process extending into dentinal tubules. They form first line of biological defense to withstand the bacterial invasion and trigger the innate and adaptive immune mechanisms of the dental pulp.[42] The activation and initiation of immune responses is possible followed by the recognition of pathogen by pulpal immune cells. The pathogenic bacteria usually have unique virulence molecular structures essential for their survival termed as 'Pathogen Associated Molecular Patterns" (PAMPs). These PAMPs are recognized by the immune system by Pattern Recognition Receptors (PRRs). PRR are the proteins expressed by the innate immune cells like macrophages, monocytes, neutrophils. PRR includes Toll Like receptor (TLR), Nucleotide-binding Oligomerization Domain (NOD) like receptor, RIG-I-Like receptors (RLRs) which when encountered by PAMP mediates the initiation of immune response along with the secretion of proinflammatory cytokines.[18]

Toll Like Receptors (TLR) have been demonstrated as a critical key for triggering innate immune response against the pathogens acting as signal transducers in pattern recognition of virulence molecules. Many in-vitro studies have detected the expression of toll like receptors on the cell membrane of cultured odontoblasts. When the odontoblasts are challenged

with exogenous noxious stimuli like lipoteichoic acid, TLR response (TLR2, TLR4) is up regulated in response to pathogens. And upon activation of TLR, the metabolic activity of odontoblasts are shifted towards the production of mediators that triggers and mediates the development of innate and adaptive immune responses (*Kawai et al 2010*).[56] TLR2 are involved in recognizing peptidoglycan, lipoteichoic acid, and lipopeptides from Gram positive bacteria while TLR4 is activated by the endotoxins like lipopolysaccharide from Gram negative bacteria (*Schwandner et al* 1999).[98] The activation of TLR results in secretion of chemokines and pro-inflammatory cytokines which in turn attracts other inflammatory cells and facilitating internal clearance by phagocytosis.[28,102]

The main feature of the innate response is that it promotes the recruitment of circulating immunocompetant blood cells like neutrophils, lymphocytes, macrophages and their migration and activation through the endothelial barrier to gain access to the site of infection to eradicate pathogens.[42]

Chemokines, the chemotactic cytokines produced by odontoblasts and fibroblasts attracts and direct the immune cells like immature dendritic cells, neutrophils, monocytes, effector and memory T lymphocytes beneath the carious lesion at the pulp-dentin interface to capture foreign antigens.[28]

In healthy tissues, leukocytes are a part of immunosurveillance to sense the invading pathogens. During inflammation, their numbers are significantly increased to detect the invaders.[29] Neutrophils, also called polymorphonuclear leukocytes are the most abundantly recruited innate immune cells. Chemotactic gradient developed inside the injured pulp due to the release of various proinflammatory mediators like chemokines, cytokines and bacterial PAMPs instruct the PMNs to leave the circulation by adhering to the endothelium lining of blood vessels and migrating out of the blood vessels to reach the site of infection. Their average lifespan is around 5-6 days. After which they undergo apoptosis and there upon removed by macrophages.[18]

Macrophages in the pulp tissue are usually derived from the monocytes in circulation. Macrophages and neutrophils are the primary phagocytes in innate immune response. The major function of macrophages is that it acts as a scavenger to eliminate antigen immune complexes and clears away the senescent cells by efferocytosis maintaining tissue homeostasis. Efferocytosis is the process of engulfing dead, necrotic or apoptopic senescent cells. Macrophages promote immunomodulation through the production of various biologically active substances like reactive oxygen species, various cytokines IL-1, IL-6, TNF-α, IFN-ɣ and growth factors.[42] They along with the dendritic cells, activates T cells and hence the onset of adaptive immunity.

Dendritic cells (DC) are the crucial antigen presenting cells in the innate and adaptive immune responses with limited phagocytic activity.

They are so called because of their surface projections giving them a dendritic appearance. DC are available as immature dendritic cells inside the pulp and are attracted to the site of infection by the chemokines released by odontoblasts.[6] Studies have confirmed the evidence of a rapid accumulation and increased populations of dendritic cells beneath the induced caries lesion (*Olshima et al 1994*).[82] These hematopoietically derived cells expressing class II molecules on their surface, detects and captures protein antigens. Following which they migrate through lymphatic system to lymphoid tissues as mature DC where they present the captured peptide fragments of antigen along with class II molecules to the naive CD4 T lymphocytes (Th0 cells). These stimulated cells migrate to peripheral tissues as memory T cells. If the tissues are again challenged by the same antigen, it is captured and presented by antigen presenting cells to the circulating memory T cells which triggers the immune response (*Pavli et al in 1993*).[89]

Natural Killer cells are activated subsequent to recognition of abnormalities in target cells. These activated NK cells produce various cytokines like IFN - ɣ which in turn activates macrophages and mediates type- 1 T cell response.[28,42]

Thus innate immune response can slow down and eliminate the bacterial invasion theoretically. However the unique location of caries bacteria seems to prevent them from being killed or eliminated by phagocytes. Hence the persistent infection and the resultant increased edema and intrapulpal

pressure due to low compliance environment leads to the activation of adaptive immunity. The unique specificity of adaptive immunity exists because B and T lymphocytes express membrane receptors that specifically recognize different antigens.[44]

Historically it has been believed that uninflammed healthy pulp tissues are devoid of lymphocytes *(Seltzer and Bender 1984).*[127] Later various immunohistochemical studies have confirmed the presence of T lymphocytes as normal essential residents in healthy pulp (*Jontell et al 1987, Hahn et al 1989.)*[82,83] The naïve T cell when activated by the matured DC inside the lymphoid tissue gets differentiated into effector CD4 T helper cells or effector CD8 T cytotoxic cells.[54]

T helper cells belonging to CD4 (cluster of differentiation marker) subset activates the functions of other immune cells (B lymphocytes, T-lymphocytes and macrophages). They are divided mainly based on the type of cytokine production into T helper 1 (Th1), T helper 2 (Th2) and Th17 cells. Th1 cells secrete proinflammatory cytokines like TNF-α, IL-2. IFN-ɣ required for the activation of macrophages and cell mediated immune response. In contrast Th2 cells produces anti-inflammatory modulators like IL-4, IL-5, IL-6, IL-10 required for the activation of immunoglobulin mediated immunity.[28]

T cytotoxic cells belong to CD8 subset and directly destroy the foreign antigens after they have been stimulated. Similar to T helper cells, T cytotoxic cells are differentiated into T cytotoxic 1 (Tc1), T cytotoxic 2(Tc2) and regulatory T (Treg) cells. Th17 sub-sets produce inflammatory cytokines for protection against fungal and bacterial microorganisms. Tc2 promotes enhanced proliferation of B lymphocytes compared to Tc1. In addition there are T suppressor lymphocytes belonging to CD8 subset inhibiting the functions of other B or T cells.[44]

Unlike T cells, usually B lymphocytes are not the normal residents of healthy pulp. Several studies have failed to identify the presence of B cells in human pulp.[39] But few immunohistochemical studies have revealed the occasional presence of B lymphocytes in human dental pulp. They produce antibodies against specific antigens and gets differentiated into plasma cells.[41]

These immunological events orchestrated in the dental pulp in response to pulpitis, ultimately results in the release of several mediators which in turn triggers a series of inflammatory events and an attempt to initiate repair which includes inflammatory chemokines, cytokines and antibodies.

Cytokines are small polypeptides secreted by leucocytes and other cells that make way for communication between the different inflammatory and immune cells thereby guiding, modifying and regulating inflammatory

and immune responses.[46] Cytokines generally operates in a network and not in a solitude fashion. They can be either inflammatory or anti-inflammatory mediators. Inflammatory cytokines comprises of interleukin IL-2, IL-6, IL-8, IFN-ɣ and tumour necrosis factor (TNF-α) that mediates and enhances inflammation, and anti-inflammatory cytokines like IL-4, IL-10, IL-13 conceals the inflammation and stimulates the healing of damaged tissues.[23] Cytokines are considered to be the outstanding markers of inflammation and its advancement to tissue necrosis (*Ashida et al. 2011).*[5] Several studies were conducted to detect and analyze the levels of a number of cytokines in different inflammatory environments to correlate with the progression of bacterial infections (*Degre 1996*).[20]

White adipose tissue is the physiological site for energy storage as lipids and supplies fuel to the body when necessary by regulating fatty acid mobilization. It consist of adipocytes, preadipocytes, macrophages, endothelial cells, fibroblasts, and leukocytes.[113] This multi-various composition of adipose tissue evidences its diverse roles, as an important mediator of metabolism and inflammation. Several studies indicate adipocytes share certain features with immune cells like production of proinflammatory cytokines (*Hotamisligil et al 1993*)[47] and fat cell precursors share certain features with macrophages like phagocytosis (*Cousin B et al 1999*).[87] Adipocytes have the ability to synthesize and secrete various active proteins called adipokines which includes inflammatory cytokines and hormone- like proteins. Example: leptin,

adiponectin, visfatin, hepcidine, TNF alpha, IL-6, resistin. Hence recently adipose tissue is considered to be an endocrine organ that can secrete hormones, peptides and cytokines (adipokines) apart from being recognized only as a fat depot.[113]

Leptin, which belongs to adipokine family, is one of the most important proteins secreted by adipose tissue. In late 1950s, two severely obese mice strains, ob/ob and db/db were the animal models used for the obesity and diabetics studies respectively. Later *Zhang and Friedman[118] in 1994* recognized and cloned the ob gene in adipose tissue. He validated that a lipostatic factor secreted by the adipose tissue were lacking in ob/ob mutation carrying mice models while the corresponding lipostatic receptor were lacking in db/db mice. He named the identified lipostatic product as "leptin" encoded by ob gene. *Greek: leptos - meaning thin*. Leptin receptor *(Ob-R)* was encoded by db gene. *Tartaglia LA et al[107]* localized leptin receptor in hypothalamus in 1995. This validates the main neuroendocrine role of leptin that it acts as a satiety factor by regulation of body weight in a central manner through its receptor Ob-Rb in the arcuate nucleus of hypothalamus by inhibition of food intake and stimulation of energy expenditure by activation of catabolic neuronal circuits and by stimulation of appetite followed by suppression of energy expenditure by activation of anabolic neuronal circuits. This 16kDa adipocyte derived hormone is mainly produced by adipose tissue and in lower levels by stomach, skeletal muscle, placenta, pituitary, bone

marrow. Leptin is one of the several keys that controls food intake. The circulating leptin levels reflects the amount of energy stored in adipose tissue and are altered by nutritional status i.e. falling with fasting and rising with overfeeding conditions.[89]

Despite the fact that adipocytes are not conventional residents of human dental pulp, when there is increased expression of Peroxisome Proliferator Activated Receptor-gamma 2 (PPARɣ2), an early adipogenic master gene and lipoprotein lipase phase-a late marker (LPL) inside the pulp, in-vitro studies showed a phenotypic conversion of human dental pulp stem cells (DPSC) to form oil red O-positive lipid containing adipocytes (*Gronthas et al in 2002*).[35] It was also shown that cultured human pulp fibroblasts when stimulated by neuropeptide Y and angiogenic growth factors, regulates the production of leptin (*El karim et al* in 2009).[22] These studies advocates the expression of leptin in human dental pulp .

The leptin receptor (Ob-R) exists in 6 alternatively spliced forms with cytoplasmic domains of different length - 4short isoforms – Ob-Ra, Ob-Rc, Ob-Rd , Ob-Rf ; soluble Ob-Re ; long isoform Ob-Rb which has the ability to transduce the signal . The four short forms of the leptin receptor are expressed by certain non-immune tissues and mediate the transport and degradation of leptin. The soluble isoform of the receptor, the OB-Re is generated by alternative splicing or by proteolytic cleavage of the membrane-bound isoform. This soluble receptor molecule is released by the leptin secreting cells

in the form of a complex with leptin. It is under the form of this leptin-leptin receptor complex that the hormone circulates in the blood since the formation of complex allows for increased half-life of leptin, modulating the action of leptin. The long form Ob-Rb, is expressed by the hypothalamus regulating appetite, body weight and bone mass. Ob-Rb is also expressed by endothelial cells, pancreatic-cells, the ovary, CD34+ hematopoietic bone-marrow precursors, monocytes or macrophages, and T and B cells. The expression of Ob-Rb by T and B cells indicates a possible role for leptin in immune-cell activation and signal transduction.[57,85]

The primary amino acid sequence of leptin shows structural and functional similarities to the long-chain helical cytokine family such as IL-6, IL-12, IL -15 and growth hormone, which is characterized by 4 helical bundles. The leptin receptor (Ob-R) also shows sequence homology to class I cytokine receptor (gp130) superfamily that includes the receptor for IL-6, leucocyte inhibitory factor (LIF), and granulocyte colony-stimulating factor (G-CSF). Hence the structure of leptin and its receptor suggests that leptin should be classified as a cytokine.[83]

Immunity requires adequate and balanced energy supply for optimal function. The availability of energy in body is reflected by the fat stored by adipocytes. Adipocytes give quality information on the bioavailability of energy required to mount sufficient immune responses. Many studies have linked the metabolisms of neuro-endocrine and immune system. In addition

there are many mediators that are common to both systems including IL1, IL-6 cytokines and TNF-α. These cytokines can cross blood brain barrier, acts on hypothalamus and modulates inflammation through hypothalamo-pituitary-adrenal (HPA) axis. Leptin is one of the mediator common to both systems that bridge immune homeostasis and metabolism, which is produced at high levels during inflammation and can be induced by other inflammatory mediators like TNF-α and IL-1, IL-6, LPS. LPS binds with the TLR-2, toll like receptors present on adipocytes stimulating the secretion of leptin and other proinflammatory cytokines.[65,67,83] Hence in addition to regulation of energy balance, leptin is a potent modulator of immune responses.

Modulation of the immune system by leptin is exerted at several levels, including development, proliferation, anti-apoptotic, maturation and activation levels. Indeed, leptin receptors have been found in almost all blood cell populations, including neutrophils, monocytes and lymphocytes.The altered production of leptin during infection and inflammatory process indicates that leptin is associated with host defense mechanisms.[10]

Since 1929, attempts have been made to correlate clinical evidence of pulpitis with microscopic findings in human teeth. Many studies have confirmed, the correlation between symptoms and histopathological changes in pulpitis to be poor and concluded that the determination of the type and extent of the inflammatory changes on the basis of symptoms is inaccurate and inconsistent (*Seltzer et al 1963*).[99] Therefore, an accurate diagnosis of the

histopathological condition of the pulp from clinical symptoms is not possible.

However, when the pulp chamber is exposed upon access opening as a part of the root canal treatment protocol, it is possible to obtain pulp tissue samples that may contain mediators reflecting the inflammatory state of the pulp. Analysis of pulpal tissue samples may provide valuable information regarding pulpal status. Dental pulp haemograms performed using pulpal blood samples collected from the teeth with severe inflammation at the exposure site showed elevated neutrophil counts (*Guthrie 1965)* [39] and elevated immunoglobulin levels IgG, IgA, IgM, elastase and prostaglandin E2 compared to normal dental pulp (*Nakanishi* et al 1995).[81] Various studies have analyzed the differences in gene expression of cytokines between the healthy and inflamed pulp tissue samples.

Previously the secretion of leptin has been reported only in adipose tissue and placenta.[79,109,114] The identification of leptin protein and its receptors in the gastric epithelium of rats *(Bado A et al 1998*)[7] and in the biopsy samples of human gastric epithelial cells *(Breidert M 1999)*[12] has paved the way for investigations on the peripheral action of leptin in dentistry. These studies however failed to detect leptin mRNA. Thereafter studies were conducted to identify the source of leptin and whether leptin is secreted or only stored in the gastric epithelium. Various researchers then affirmed the presence of leptin in saliva and leptin mRNA synthesis from salivary glands and oral mucosa *(Groschl.M et al 2009)* [36] and expression of leptin in the

ameloblasts, odontoblasts and few dental papilla cells in rat tooth germs *(Ide et al 2011)*[51]. Leptin has also been reported in healthy and inflamed gingival tissues *(Johnson et al in 2001)*[52] and chronic elevation of leptin increases the risk for acute myocardial infarction *(Gundala et al 2014).*[38]

Later investigations on periapical lesions collected during periapical surgery revealed the presence of leptin.[69,70] *Martin Gonzalez et al* in *2013* has conducted an in-vitro study to investigate the presence of leptin in healthy and inflamed human dental pulp tissue collected from the extracted teeth samples and concluded the presence of leptin in pulp samples with elevated expression of leptin in the inflamed pulp tissue samples than in healthy tissues.

However the behavior of leptin and its role in pulpal inflammatory cascades in a biological system that would be clinically relevant has not been assessed. Also there is no literature available comparing and evaluating the role of leptin in acute and chronic pulpal pathological conditions. Hence, the present study was undertaken to describe the possible expression of Leptin in human dental pulp, and to evaluate its role in acute (symptomatic) and chronic (asymptomatic) pulpal pathosis. In this study, the framed hypothesis attempts to show that leptin is expressed in all groups and there is no difference in its expression between the groups.

In the present study, human pulp tissue samples were collected from mandibular molar teeth from a total of 30 patients who gave their written

informed consent to donate their pulp tissue. The groups were divided into acute pulpal pathosis (symptomatic n=10), chronic pulpal pathosis (asymptomatic n=10) and healthy samples (n=10) classified according to the American Association of Endodontists (AAE) guidelines (2012)[33].

Normal Pulp - A clinical diagnostic category in which the pulp is symptom-free and normally responsive to pulp testing.[33]

Reversible Pulpitis - A clinical diagnosis based on subjective and objective findings indicating that the inflammation should resolve and the pulp returns to normal.[33]

Symptomatic Irreversible Pulpitis (acute) - A clinical diagnosis based on subjective and objective findings indicating that the vital inflamed pulp is incapable of healing. Additional descriptors: Lingering thermal pain, spontaneous pain, referred pain.[34]

Asymptomatic Irreversible Pulpitis (chronic) - A clinical diagnosis based upon subjective and objective findings indicating that the vital inflamed pulp is incapable of healing. Additional descriptors: No clinical symptoms but inflammation produced by caries, caries excavation, trauma, etc.[33]

In group 1 and group 2 the collection of pulp tissue was done from the patients during the endodontic treatment of mandibular molars diagnosed with acute and chronic pulpal pathosis respectively, with the help of ISO size #15

H-file, Dentsply Maillefer, Switzerland (figure 1) and ISO size #15, #20 Broaches, Pfiffer Dent, France (figure 3) after the pulp chamber was carefully deroofed with a high-speed hand piece. Diagnosis of acute (symptomatic) and chronic (asymptomatic) pulpal pathosis was based on subjective history, clinical findings, periapical radiographs and pulp sensibility testing (thermal tests and electric pulp test). The collected pulp tissue samples were stored in labeled Eppendorf tubes containing ethanol and kept at -80°C deep freezer (figure 6) until use in accordance with previous studies.[72]

In group 3, healthy pulp tissue samples were collected from freshly extracted, intact and caries free mandibular molars which were confirmed with the help of radiographs (figure 2). Extraction was done as the experimental teeth were impacted or ectopically positioned under local anesthesia (Lignox 2% with adrenalin 1:80,000) without any complications. Immediately after extraction, each tooth was washed with 5.25% sodium hypochlorite to exclude the remnants of periodontal ligament and sectioned axially at the level of CEJ by using a diamond rotary disc in dental hand piece with adequate water coolant. The pulp tissue was removed gently by using an endodontic broach and transferred to labeled Eppendorf tube containing ethanol (figure 5) immediately and kept at -80°C until use.

In the present study, the expression of leptin in the collected acute, chronic and healthy pulp samples was performed by quantitative real time polymerase chain reaction (PCR). Gene expression is the process by which the

genetic information encoded in a gene is transcribed into a functional product (usually mRNA or proteins). Real time PCR is a recently introduced sensitive, reproducible and precise technique for the real time monitoring of reaction process and to quantify gene expression.

The quantification of gene in real time PCR is done by 2 methods, absolute quantification and relative quantification. Absolute qPCR assays enables the quantification of single target template and the final result is expressed as an absolute value (e.g. copy numbers/ml). This analysis is based on the construction of a standard curve from PCR amplified products with the known template concentration. Relative Quantification enables the comparison of two different genes in a single sample and the final result is expressed as a ratio of both these genes. Usually one gene is the target gene and other is the reference gene kept as an endogenous control for normalization.[75]

Gene expression studies are routinely used to determine the variations in the expression of a target gene in diseased state or under the course of treatment relative to a defined healthy or untreated state. Hence relative quantification is the most commonly used method to compare the changes in amplification of the target gene expression under different states through the utilization of reference gene or internal control.[21]

In our present study, Relative quantification of Leptin was performed in the collected pulp tissue sample with the help of Real Time PCR machine, Light Cycler ® 480 Real-Time PCR System, Roche, Germany (figure 24). Leptin is the target gene. Usually Housekeeping genes are kept as internal

control or reference gene since they have different sequences compared to target and show a consistent level of expression in all the samples irrespective of the disease process. Cyclophilin is kept as the reference housekeeping gene in accordance with previous studies (Martin et al).[70,72]

In this study, the relative quantification of leptin normalized to cyclophilin in real time PCR (qRT-PCR) includes the following steps. The first and the most important step in analyzing gene expression, is obtaining an intact RNA. The tissue has to processed immediately to harvest RNA or has to be stabilized in a proper storage medium to preserve the integrity of RNA. Total RNA was isolated from pulp tissue samples using QIAamp RNA extraction Kit, Qiagen (figure 7) according to the manufacturer's recommendations. The pulp tissue samples were homogenized using Sterile Sticks (figure 12, 13). Quantitative and qualitative evaluation of extracted Total RNA was performed on NanoDrop™ 2000c Spectrophotometer, Thermo Scientific™, US (figure 17,18) at 260 and 280nm. Secondly, 5µl of the extracted Total RNA was reverse transcribed to first stranded complementary DNA (cDNA) by reverse transcriptases in Thermocycler, Applied Biosystems™ (figure 22,23) at 25°C for 5 min , 42°C for 60 min and finally at 70°C for 5 min using RevertAid First Strand cDNA Synthesis kit , Thermo Scientific™, US (figure 19) according to the protocol attached. cDNA were used as templates for amplification in polymerase chain reaction using Power SYBR Green PCR master mix according to the manufacturer's instruction using specific primers for target and reference gene. Quantitative real-time

PCR analysis was performed in Roche, LightCycler® 480, Germany using the primers for leptin and cyclophilin based on the sequences provided by the National Center for Biotechnology Information Gen Bank database:(figure 25)

Leptin	Forward	5-GAACCCTGTGCGGATTCT- 3
	Reverse	5CCAGGTCGTTGGATATTTGG-3
Cyclophilin	Forward	5-CTTCCCCGATACTTCA-3
	Reverse	5-TCTTGGTGCTACCTC-3

In the present study, the relative quantification and data analysis where performed by ΔΔCT-Method according to the instructions of the Roche, LightCycler® 480 real time software. Samples were used in triplicates for data accuracy. Crossing point (CP) or crossing threshold (CT) is the cycle at which fluorescence from amplification attains a defined threshold and is first detected. Each sample requires different number of cycles to reach the crossing point, depending on the initial concentration of DNA in the sample. The real time quantification was monitored by measuring the increasing fluorescence caused by the binding of SYBR green dye to double standard DNA at the end of each amplification cycle[102] i.e., Cp values are correlated for the efficacy of amplification (*Piantoni et al 2008).*[91]

On analyzing the data, the results of the present study revealed that all the collected human pulp tissue samples (n=30), have presented the expression of leptin (figure 27). Martin et al[72] in 2013 has also described the expression of leptin in human pulp tissue. The threshold cycle values for the expression of target gene leptin in acute pulp tissue samples ranged between 38.29 and 41.23 (table1, graph1). The threshold cycle values for the expression of leptin in chronic pulp samples ranged between 36.84 and 41.65 (table 2, graph 2). Similarly the threshold cycle values for the expression of leptin in healthy dental pulp samples ranged between 30.21 and 38.29 (table 3, graph 3). The expression of leptin in all pulp tissue samples has confirmed the first hypothesis. While comparing the mean crossing point values (table 4, graph 4) (figure 28,29) for target gene leptin expression between acute (39.854 ± 2.469) and healthy pulp tissue samples (33.302 ± 2.260), there is statistically significant difference between both the groups with p value of 0.000 ($p<0.05$) (table 5, graph 5) . The data also indicates that while comparing the chronic (37.878 ± 1.498) and healthy pulp tissue samples (33.302 ± 2.260), there is statistically significant difference between both the groups with p value of 0.000 ($p<0.05$) (table 5, graph 5) (figure 28, 29). Hence the null hypothesis is rejected. The result indicates that the leptin expression in acutely inflamed pulps is increased by 19.67% compared to that of healthy pulp tissue and in chronically inflamed pulps the expression is increased by 13.74% compared to healthy samples (table 5, graph 5). These results are in accordance with previous studies by Martin et al[72] (2013) that the relative leptin mRNA

expression in inflamed pulps is almost increased by 50% compared to normal pulps. There is a statistically significant difference while comparing the threshold cycles for target leptin gene expression in acute (39.854 ± 2.469) and chronic (37.878 ± 1.498) pulp tissue samples with a p value of 0.001 ($p<0.05$) (table 5, graph 5).

On analyzing the results for the reference housekeeping gene cyclophilin, threshold cycle values for acute pulp samples ranged between 37.653 and 48.033, for chronic pulp samples ranged between 36.573 and 39.236, for healthy pulp samples ranged between 36.07 and 38.86 (table 6, graph 6) (figure 30,31). While comparing the mean values for Cyclophilin expression between acute (39.38 ± 3.618), chronic (38.148 ± 1.189) and healthy (37.438 ± 0.99), there was no any statistical difference in the expression of Cyclophilin among all the three groups with a p value of 0.230 ($p>0.05$) (table 7, graph 7)

In the present study, the expression of leptin in all the study samples reveals that leptin is secreted by dental pulp stem cells (DPSC) encountering adipogenic differentiation irrespective of the nature of pulp (either healthy or inflammed). *Koyama et al*[59] *in 2009, Um et al*[109] *2011* also have previously observed the release of leptin in differentiated DPSC cultured from healthy and carious tooth.

In this study, the expression of leptin in acute and chronic pulp tissue samples is significantly higher when compared to healthy samples. The up-regulation of leptin protein in inflamed pulp than normal pulp suggests that leptin has a role in the process of pulpal inflammation (pulpitis). Several authors have reported that leptin receptors are present in almost all immune cells and in general, leptin acts as a proinflammatory cytokine promoting the production of various cytokines from the immune cells during inflammation (Fantuzzi et al 2000, Cava et al 2004, Matarese et al 2005, Lord 2006, Otero et al 2006).[27,64,75,67,83]

Many in-vitro studies have shown Leptin activates the proliferation of human circulating monocytes. *Zarkesh Esfahani*[116] *et al* in 2001 showed leptin regulates increased expression of monocyte surface markers like CD25 (IL2 receptor), CD69, CD38, CD71 and also activates Human Leukocyte Antigen (HLA) , CD11b, CD11c markers expression present already on the resting monocytes and macrophages . It stimulates secretion of pro-inflammatory cytokines like TNF-α in early stage and IL 6 and IL 12 at later stages and there is also an up regulation of phagocytic function of macrophages. Also by measuring production of free radicals, leptin activates the oxidative stress in monocytes and macrophages. Finally, leptin might act as a potent chemo attractant and induces leptin mediated chemotactic responses in monocytes and macrophages.

Similar to monocytes, leptin enhances the longevity of dendritic cells with augmented expression of specific surface molecules CD1a, CD80, CD86, CD83. There is increased production of several cytokines IL-8, IL-6, IL-12, TNF-α by dendritic (DC) cells, but decreased production of MIP-1a. *Mattioni et al*[76,77,78] in 2005, 2008, 2009 showed in his studies that leptin is included in dendritic cell's maturation by enhanced expression of specific surface marker CCR7 and favoring its dynamics by their chemotactic responses. Short form of leptin receptor is expressed in human polymorphonuclear neutrophils. The effects of leptin are mediated in human PMN through indirect and direct mechanisms as well. Leptin activates the release of cytokines like TNF-α by monocytes, which in turn promotes the chemotaxis of neutrophils and release of intracellular oxygen radicals like hydrogen peroxide and superoxide anion. Thus leptin plays as a 'survival cytokine' protecting neutrophils from apoptosis.[115] Leptin influences almost all cellular activities of natural killer(NK) cells including differentiation, proliferation, activation and cytotoxicity by up- regulation of IL-12 production and reducing IL-15 in NK cells at transcription levels . Both short and long forms of leptin receptor are expressed in human NK cells. The stimulation of IL-1 production from monocytes and macrophages by leptin, also indirectly activates the natural killer cells.[119]

Leptin induces morphological and functional changes in dendritic cells and induces T cell activation. Leptin also modulates cytokine production by

T cells towards type 1 cytokines (eg. TNF, IL-12) by polarizing the differentiation of T cells to shift towards Th1 response and suppressing the Th2 cytokines production (Lord et al 1998).[66] Kiguchi et al[58] in 2009 observed that leptin also stimulates the expression of CC-Chemokine ligand through activation of JAK2-STAT3 signaling pathways in cultured murine macrophages which is essential for recruiting dendritic cells and memory T cells into the site of inflammation. This signaling pathway has also been identified in human dental pulp by Zhou et al[120] in 2011. These findings suggests that the relative increased expression of leptin in inflamed pulp tissue samples, regulates increased expression of pro-inflammatory cytokines by the immune cells and acts as a potent inflammatory mediator.

In this present study, the results showed that there is a significant increase in the expression of leptin in acute pulp tissue samples compared to chronic pulpal pathosis samples. The innate and adaptive immune systems are interconnected so that in response to pathogenic stimuli, the pulp tissue initially mounts innate immune response, followed by adaptive immune response in a coordinated manner.

During the initial phase of local acute inflammatory reaction, there is recruitment of increased numbers of innate immune cells and production of cytokines. Innate immunity lasts only for several hours to few days. In the late phase of acute response when innate immunity fails to eliminate the noxious stimuli, adaptive response gets activated.[95] Leptin also modulates the immune

cells especially macrophages in a dose dependent fashion and there is increased production of the pro-inflammatory cytokines like IL-2, TNF-α, IL-12. These type-1 cytokines orchestrate strong cellular immunity and suppresses the synthesis of type-2 cytokines[121]. In addition, leptin switches the differentiation of T cells towards Th1 priming and induces production of type-1 cytokine profile, suppressing type- 2 profile.[83]

Whereas in case of chronic inflammatory reaction, various immunohistochemical studies have reported the increased numbers of B cells (Jontell et al 1998, Hahn et al 2007)[54,41] and increased type 2 cytokine profile. In chronic persistent infection due to the prolonged exposure to microorganisms and noxious byproducts, the resolution phase becomes disturbed and disorganized, and there appears a shift from Th1 to Th2 profile. These predominance of type 2 cytokines (eg. IL-4, IL-10) suppresses the activation of macrophages and consequently suppresses type 1 cytokine profile. They stimulate the proliferation of B cells into plasma cells.[44] Sanna et al[97] 2003 in his study also observed similar finding that decrease in serum leptin levels during acute starvation promoted Th2 mediated cytokine switch. Hence the skewing of cytokine profile towards Th2 and activation of B cells explains the decrease in relative leptin expression in chronic pulpal samples compared to acute pulpal pathosis. Gualillo et al[37] in 2000, Popo et al[92] in 2005 have also reported that acute infection, sepsis and inflammatory mediators stimulate leptin synthesis, whereas in contrast chronic stimulation

with inflammatory cytokines causes leptin suppression. In addition, the inappropriate and unregulated accumulation of leukocytes and their prolonged survival span, there is a continuous production of inflammatory mediators, finally leading to the complete necrosis of the tissue. Despite the secretion of protective cytokines, it may be too late to reverse the damage in chronic pulpitis. The decreased expression of leptin in chronic pulpal pathosis can also be due to partial necrosis of the pulp tissue.[46]

This is the first ex-vivo study to compare the expression of leptin in healthy and inflamed human pulp tissue samples in a biological system that would be clinically relevant and also the first study to assess the expression of leptin in acute and chronic pulpal samples. The present study could have been extended to include the subgroups based on gender, weight, duration of symptoms, type of tooth and the sample size could have been extended to include larger size, and to access the association of leptin on macrophages in pulpal inflammation.

The objective of the present study is to gain a clear outlook in the intricate molecular interactions between leptin and immune systems during pulpal inflammatory process. In the present study, leptin is expressed in both healthy and inflamed human dental pulp tissue samples regardless of their inflammatory states may confirm the immunomodulator role of the adipocyte gene leptin in pulpal inflammation. In addition, the result of the study indicates the expression of leptin is highest in acute pulpal conditions followed

by chronic pulpal samples and the lowest expression in healthy samples. This suggests a probable relationship between the leptin abundance and different pulpal conditions categorized based on subjective and objective signs and symptoms along with the radiographic examination. Zehnder et al in 2011 have advocated the evaluation of molecular markers in dentinal fluid as indicators of pulpal inflammation.[117] This suggests that leptin could potentially serve as promising bio-markers for pulpal inflammation in near future. This necessitates further studies in future to investigate the levels of leptin biomarker in clinical chair-side tests by noninvasive techniques without actually collecting the pulp samples.

Summary

SUMMARY

In the present ex-vivo study, the aim was to evaluate the expression of leptin in the healthy and inflamed human dental pulp samples by quantitative real-time PCR assay and to compare and analyze the variations in the relative expression of leptin in healthy, acute and chronic pulpal pathosis groups. A total of thirty mandibular molars of healthy subjects were included in the study. All the teeth were verified by digital radiographs and pulp sensibility tests along with the clinical history of signs and symptoms and divided into three groups. Group 1- acute pulpal pathosis (n=10), Group 2- chronic pulpal pathosis (n=10) and Group 3- healthy pulp (n=10). In group 1, group 2 the pulp samples were collected after access opening during endodontic treatment under local anaesthesia (1:80,000 adrenaline) using broach and H-files. In group 3, caries and restoration free impacted molars, immediately after extraction were sectioned axially at CEJ level with adequate water coolant and pulp tissues were collected similar to the other groups. The collected samples were stored in labeled Eppendorf tubes containing ethanol in -80°C deep freezer. Total RNA was isolated from the human dental pulp tissue samples with QIAamp RNA extraction Kit, Qiagen according to manufacturer's recommendations. The concentration of RNA was assessed in nanodrop spectrophotometer at 260nm and 280nm. Then for the synthesis of cDNA, total RNA was reverse transcribed using RevertAid First Strand cDNA Synthesis kit, Thermo Scientific™ under optimal thermal conditions in thermocycler: 25°C for 5 min, 42°C for 60 min and 70°C for 5 min.

The cDNA of the target leptin gene and the reference gene cyclophilin were amplified with specific primers and Power SYBR™ Green PCR master mix. Quantitative Real time PCR assay was done in Roche LightCycler ® 480 Real-Time PCR System, Mannheim, Germany. The temperature cycles were as follows –preheating at 50°C for 2 min, heating at 95°C for 10 min. Eventually 41 amplification cycles of PCR reactions were carried out under the following conditions - denaturation for 15 s at 95°C, annealing for 1 min and extension at 58°C. All the samples were run in triplicates and equal amount of PCR products were loaded into each well. The amplification curves were generated by LightCycler ® 480 PCR software and the data were subjected to statistical analysis using Statistical Package for Social Sciences software (SPSS version 22, USA) using One- way ANOVA and post HOC TURKEY tests.

Conclusion

CONCLUSION

Under the conditions of the present ex-vivo study and within its limitations, the following conclusions can be drawn:

1. The pro-inflammatory cytokine leptin is expressed in all the collected healthy and inflamed human dental pulp tissue samples regardless of their inflammatory states.
2. The expression of leptin is significantly upregulated in inflamed (acute and chronic) pulp samples compared to healthy samples.
3. Among the inflamed pulpal samples, the acute pulpal pathosis group showed elevated leptin expression compared to chronically inflamed pulp samples which is statistically significant.

This study shows the dramatic alteration of the proinflammatory cytokine, leptin in different inflammatory conditions of pulp highlighting the molecular events that regulate the increased production of leptin. In addition this study identifies a potential novel biomarker for future research that may probably have tremendous impact on preventive medicine globally.

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Annexures

ANNEXURE – I

CONSENT FORM 1

You are being asked to participate in a research study. This form provides you information about this study. Please read the information below and contact the person in charge of this research (Principal Investigator) regarding anything you don't understand before deciding whether or not to participate in the study. Your participation is entirely involuntary

TITILE OF RESEARCH STUDY:

Expression of leptin in human dental pulp and its role in acute and chronic pulpal pathosis

PRINCIPAL INVESTIGATOR:

Dr. P.M.Vikhashini,
Post Graduate Student,
Department of Conservative dentistry and Endodontics
Ragas Dental College and Hospital.

The purpose of this study is to determine the presence of leptin gene and its association in dental pulp. This study involves collection of pulp tissue from the participants. Endodontic treatment results in the removal of pulp tissue (nerves and blood vessels) from inside of the tooth and sealing the space with a filling material. The pulp tissue will be collected as a part of the endodontic treatment recommended for your tooth. The collected samples will be analyzed for leptin.

1. This collection is painless with no physical risks.
2. Data from this study does not affect or influence your present treatment.
3. The procedure carries no financial burden on your part

I, …….. S/O,…….. D/O…… W/O, ……., aged about …… years, residing at ……., do hereby solemnly and state as follows.

I was informed and explained about this research in the language known to me. I was also informed and explained, that the results of the test will not be revealed to public. I give my consent after knowing the procedure, alternatives and full consequences of the study and, I had been given sufficient time to take my decision. I undertake to cooperate with the doctor for the study.

Signature of the patient Date:

Signature of the attendant Date:

Signature of the Doctor Date:

CONSENT FORM 2

You are being asked to participate in a research study. This form provides you information about this study. Please read the information below and contact the person in charge of this research (Principal Investigator) regarding anything you don't understand before deciding whether or not to participate in the study. Your participation is entirely involuntary

TITILE OF RESEARCH STUDY:

Expression of leptin in human dental pulp and its role in acute and chronic pulpal pathosis

PRINCIPAL INVESTIGATOR:

Dr. P.M.Vikhashini,
Post Graduate Student,
Department of Conservative dentistry and Endodontics
Ragas Dental College and Hospital.

The purpose of this study is to determine the presence of leptin gene and its association in dental pulp. This study involves collection of pulp tissue from the extracted tooth of the participants. The pulp tissue (nerves and blood vessels) will be collected from the tooth after removal since extraction is the recommended treatment for your tooth. The collected samples will be analyzed for leptin.

1. This collection is painless with no physical risks.
2. Data from this study does not affect or influence your present treatment.
3. The procedure carries no financial burden on your part

I, …….. S/O,…….. D/O…… W/O, ……., aged about …… years, residing at ……, do hereby solemnly and state as follows.

I was informed and explained about this research in the language known to me. I was also informed and explained, that the results of the test will not be revealed to public. I give my consent after knowing the procedure, alternatives and full consequences of the study and, I had been given sufficient time to take my decision. I undertake to cooperate with the doctor for the study.

Signature of the patient Date:

Signature of the attendant Date:

Signature of the Doctor Date:

ANNEXURE – II

ஒப்புதல் படிவம்

நீங்கள் ஒரு ஆய்வில் பங்கேற்க கேட்டுக்கொள்ளப்படுகிறீர்கள். இந்த படிவம் அந்த ஆய்வு பற்றிய தகவலை தங்களுக்கு தெரிவிக்கும். இதில் உள்ள தகவலை படித்து தங்களுக்கு ஏதாவது சந்தேகம் இருந்தால் இந்த ஆய்வுக்கு பொறுப்பேற்று இருக்கும் மருத்துவரை அணுகிய பிறகே இந்த ஆய்வில் பங்கேற்குமாறு கேட்டுகொள்ளப்படுகிறது. தங்களுடைய பங்கேற்பு யாருடைய நிர்பந்ததுக்கும் உட்பட்டதல்ல.

ஆய்வின் தலைப்பு

பல்லில் இருந்து எடுக்கப்படும் தொற்று திசு மற்றும் லெப்டின் என்ற மரபணு பற்றி அறிதல்

தலைமை ஆய்வாளர்

பி. மு. விக்காஷினி
முதுகலை பல் மருத்துவ படிப்பு
ராகாஸ் பல் மருத்துவ கல்லூரி
சென்னை-119.

இந்த ஆய்வின் நோக்கம் லெப்டின் என்ற மரபணு மற்றும் பல் இருந்து எடுக்கப்படும் தொற்று திசு உள்ள தொடர்பை மேலும் நன்றாக புரிந்துகொள்வதேயாகும் பங்கேற்பாளர்களிடம் இருந்து பல் திசு பெறுவது இந்த ஆய்வின் ஒரு பகுதியாகும். வேர் சிகிச்சை என்பது பற்களின் இயற்கை வேர்களை நீக்கி, செயற்கை வேர்கள் பொருத்தபடும்.

உங்கள் வேர்சிகிச்சையின் ஒரு பகுதியாக நிக்கபடும் தொற்று பல் திசு சேகரிக்கபடும். சேகரிக்கப்பட்ட தொற்று பல் திசுவில் லெப்டின் மரபணு உள்ளதா என்று ஆய்வு செய்யப்படும்.

- தாங்கள் தொற்று திசு தருவதால் எந்த வித வலியோ, உடலுக்கு எந்தவித ஆபத்தும் கிடையாது.
- இந்த ஆய்வில் இருந்து பெறப்படும் தகவலால் உங்களுடைய சிகிச்சைக்கு எந்தவிதத்திலும் பாதிப்பு எற்படாது.
- இதில் பங்கேற்பதால் உங்களுக்கு எந்தவித பொருளாதார செலவும் கிடையாது.

நான் ..... வயது. ..... S/o, W/o, D/o, ...இ ..... வசிக்கும் நான் உறுதியோடு பின்படுமாறு கூறுகிறேன். இந்த ஆய்வு பற்றிய தகவலை எனக்கு தெரிந்த மொழியில் எனக்கு எடுத்துரைத்தார்கள். இந்த ஆய்வின் தனிப்பட்ட முடிவை பொதுமக்களுக்கு வெளிப்படுத்தமாட்டார்கள் என்று தெரிவித்துள்ளார்கள். இந்த ஆய்வின் முழுத்தகவலையும், மாற்று சிகிச்சைகளையும் தெரிந்த பிறகே நான் மருத்துவருடன் ஒத்துழைக்க ஒப்புக்கொள்கிறேன்.

இந்த ஆய்வில் நான் ஒப்புதல் அளிக்க தகுந்த நேரம் அளிக்கப்பட்டுள்ளது.

நோயாளியின் கையெழுத்து நாள்

நோயாளியின் உடன் இருப்போர் கையெழுத்து நாள்

மருத்துவரின் கையெழுத்து நாள்

ANNEXURE – III

RAGAS DENTAL COLLEGE & HOSPITAL
(Unit of Ragas Educational Society)
Recognized by the Dental Council of India, New Delhi
Affiliated to The Tamilnadu Dr. M.G.R. Medical University, Chennai
2/102, East Coast Road, Uthandi, Chennai - 600 119. INDIA.
Tele : (044) 24530002, 24530003-06. Principal (Dir) 24530001 Fax : (044) 24530009

TO WHOMSOEVER IT MAY CONCERN

Date: 6.1.2018

Place: Chennai

From
The Institutional Review Board,
Ragas Dental College & Hospital,
Uthandi,
Chennai – 600119.

The dissertation topic titled **"EXPRESSION OF LEPTIN IN HUMAN DENTAL PULP AND ITS ROLE IN ACUTE AND CHRONIC PULPAL PATHOSIS"** submitted by **Dr. VIKHASHINI. P.M** has been approved by the Institutional Review Board of Ragas Dental College & Hospital.

Dr. N.S. AZHAGARASAN, M.D.S.,
Member Secretary,
Institutional Review Board,
Ragas Dental College & Hospital,
Uthandi,
Chennai – 600 119.

RAGAS DENTAL COLLEGE Chennai

ANNEXURE – IV

URKUND

Urkund Analysis Result

Analysed Document: Viks thesis.pdf (D35040366)
Submitted: 1/26/2018 9:06:00 AM
Submitted By: vikhashinidr@gmail.com
Significance: 4 %

Sources included in the report:

thesis print final.docx (D34109367)
Divya Thesis Final.pdf (D34338844)
https://en.wikipedia.org/wiki/Leptin
http://europepmc.org/articles/PMC3328736
http://onlinelibrary.wiley.com/doi/10.1111/iej.12009/abstract
https://personal.us.es/segurajj/documentos/CV-Art%20JCR/72-IEJ-Leptin-2012.pdf
http://www.jced.es/congress/2014/jced.17643799.pdf
http://www.jced.es/congress/2014/jced.17643833.pdf

Instances where selected sources appear:

30